DETECTION, DIVERSITY, AND ACTIVITY OF ANAEROBIC AMMONIUM OXIDIZING BACTERIA (ANAMMOX) IN THE CAPE FEAR RIVER ESTUARY

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ABSTRACT

Anammox is a new pathway in the nitrogen cycle, in which NH_4+ oxidiation is coupled to NO_2- reduction to produce N_2. Anammox was first discovered in a wastewater treatment plant in 1995, and the process was attributed to bacteria that branch deeply in the bacterial genus *Planctomycetes*. Since this initial finding, anammox bacteria have been detected in anoxic marine sediments, oxygen minimum zones of the oceanic water column, estuaries, arctic sea ice, and even a freshwater lake. Activity measurements have further revealed that these bacteria can contribute significantly to the nitrogen removal process. This chemolithotrophic process is a new loop in the biogeochemical nitrogen cycle that was previously unknown. Prior to the discovery of anammox, denitrification was considered to be the only process responsible for nitrogen removal via N_2 gas production. Little is known about the genetics, ecology, and physiology of this organism. Therefore, this study was an attempt to gain better understanding in the community structure of anammox bacteria and their activity related to environmental parameters such as, ammonium, nitrate, and salinity levels. The Cape Fear River Estuary was chosen as the study site. A combination of molecular tools and stable isotope probing technique was employed to study anammox bacteria in the Cape Fear River Estuary. Anammox bacteria were identified at all three sampling sites, and each site had distinct community structure. Salinity and ammonium levels were positively correlated with diversity. Although, no correlation could be established between environmental parameters and activity levels, there did seem to be a link between community structure and activity levels.

CHAPTER 2 ABSTRACT

Anaerobic ammonium oxidation (ANAMMOX) is a new nitrogen removal process that combines ammonium and nitrite to yield nitrogen gas under anaerobic conditions. This mechanism was first discovered in wastewater systems, but has been subsequently recognized to be significant in marine, estuary, and freshwater environments as well. However, the diversity and distribution of anammox bacteria in estuarine sediments are not fully understood related to environmental parameters such as salinity and levels of ammonium and nitrate. Therefore, molecular techniques were developed to detect anammox bacteria only and to examine their diversity and distribution in Cape Fear River estuarine sediments. Sediment samples were collected from three sites (NAV, M61, M54) that vary in salinity, nitrate, and ammonium levels. Unique community structure composed of at least four different anammox bacteria (*Brocadia, Kuenenia*, *Scalindua,* or *Anammoxoglobus)* was identified at each sampling site. The most freshwater sampling site (NAV) only had *Brocadia*-like organisms present, but the sampling site of highest salinity (M54) was dominated by *Scalindua* organisms. The site of intermediate salinity (M61) had a community with three different types of anammox bacteria (*Scalindua, Brocadia*, and *Kuenenia*) present, but it was dominated by *Kuenenia* organisms. Seasonal variation of anammox communities was observed with T-RFLP analysis, which detected higher diversity in anammox organisms in the fall compared to the spring. The differences in environmental parameters, including salinity and ammonium, among the sampling sites apparently affected anammox community structure. The number of operational taxonomic units per site increased downstream with salinity. This is the first time *Brocadia, Anammoxoglobus,* and *Kuenenia* organisms have been detected in an estuarine environment. The Cape Fear River Estuary, with its natural salinity gradient, proved to be a rare and exclusive study site for

anammox bacteria; these observations could provide a better understanding of niche characteristics of anammox bacteria in nature.

CHAPTER 3 ABSTRACT

Potential rates of anammox and denitrification reactions were measured at three sampling sites (M54, M61, and NAV) of the Cape Fear River Estuary using an isotope ratio mass spectrometer (IRMS) with anaerobic ^{15}N IPT experiments. Rates were derived from a series of sediment incubations that were spiked with one of the following three treatments: $^{15}NO_3$- plus $^{14}NH_4^+$, $^{14}NO_3$- plus $^{15}NH_4^+$, and $^{15}NH_4^+$ only. Activity measurements were conducted in order to find correlations between anammox activity by communities present at each of the sampling sites in the Cape Fear River Estuary and the environmental parameters of salinity, nitrate, and ammonium at each station. The highest anammox activity was observed in site M54, which had the highest salinity, highest ammonium levels, and lowest nitrate levels. It contributed to 15.5% of total N_2 production, which was nearly double the contribution of anammox N_2 production at site NAV. Site NAV with the lowest salinity, lowest ammonium levels, and highest nitrate levels had lower anammox activity than M54, with anammox contributing to 8.6% of total N_2 production. Anammox activity was the lowest in site M61, which had intermediate levels of these environmental parameters. Anammox activity only contributed to 4.3% of total N_2 production in site M61. Denitrification activity was much greater in all sampling sites than anammox activity. No clear correlations were established between environmental parameters and anammox or denitrifier activity. There was some correlation however with the anammox community structure and anammox activity (i.e., rates). Site M54, the site with the highest anammox activity, has *Scalindua*-like bacteria as the dominant anammox bacteria. *Scalindua* species are predominately found in natural systems, in contrast with other anammox bacteria that are usually detected in wastewater treatment plants. *Scalindua* organisms, therefore, may be

preferentially adapted to the variable estuarine conditions present in the Cape Fear River Estuary and this adaptation is manifested as higher activity.

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DEDICATION

I would like to dedicate this thesis to my father, Jimmy Reed, who constantly reminded me how important it is to be serious about school and receive a good education.

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CHAPTER 1. INTRODUCTION TO ANAEROBIC AMMONIUM OXIDIZING (ANAMMOX) BACTERIA

The Biogeochemical Nitrogen Cycle

Nitrogen is an element essential to all life; it consists in proteins, nucleic acids, and many other cellular components (Ehrlich, 1990; Madigan & Martinko, 2006). The biogeochemical cycle of nitrogen is complex with valence states of nitrogen from –3 to +5, and it offers a rich variety of important biotic and abiotic processes that involve many important compounds in the gas, liquid, and solid phases (Rosswall, 1981; Jaffe, 1992). Although abundant on earth, 96% of nitrogen is found in the lithosphere and is not involved in the biogeochemical cycle of nitrogen. Bioavailablity of nitrogen is dependent on microorganisms involved in the nitrogen cycle (Rosswall, 1981). Inorganic forms of nitrogen including ammonia, nitrite, and nitrate play significant roles in geomicrobial processes, which control primary and secondary productions in aquatic environments (Ehrlich, 1990). The key processes in the nitrogen cycle are nitrogen fixation, assimilation, nitrification, dissimilatory nitrate reduction to ammonia (DNRA), ammonification, and denitrification (Figure 1) (Rosswall, 1981).

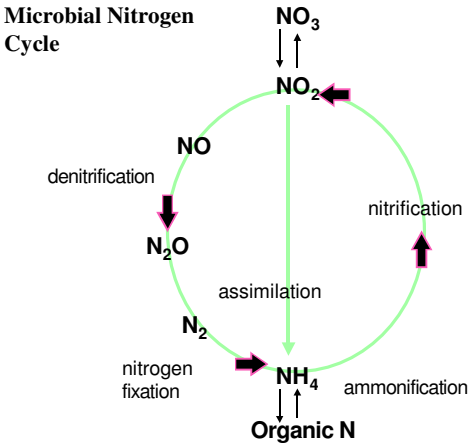

Figure 1. Diagram of the traditional microbial nitrogen cycle

Nitrogen fixation is the primary biological process to bring back nitrogen into the biosphere. It is mediated by the O_2-sensitive nitrogenase enzyme, which catalyzes reduction of nitrogen gas (N_2) to ammonia (NH_3) (Jenkins et al., 2004). It is generally considered that nitrogen limits productivity of phytoplankton in the oceans, and in the oceanic biome there are few known N_2 fixers relative to the numbers of N_2 fixers found in lakes and estuaries (Zehr & Ward, 2002). N_2 fixation occurs in a variety of prokaryotic organisms, both symbiotic and free-living. Bacteria such as those in the genera *Bradyrhizobium* and *Rhizobium* establish a specific symbiotic relationship with legume host plants. These bacteria prompt formation of new organs known as root nodules. In these root nodules, nitrogen-fixing bacteria reduce atmospheric nitrogen to ammonia and supply the host plant with combined nitrogen (Soberon et al., 1999). These root nodules allow the nitrogenase enzyme to maintain an anaerobic environment. Legumes, such as soybeans, are commonly used in crop rotations because this symbiotic relationship of the plant roots with nitrogen-fixers replenishes nitrogen in the soil. The most common nitrogen-fixing organisms in the ocean are cyanobacteria, namely the filamentous nonheterocystous cyanobacterium *Trichodesmium* (Capone et al., 1997; Orcutt et al., 2001) and heterocyst-forming cyanobacterial symbionts of diatoms (Carpenter et al., 1999; Villareal & Carpenter, 1989). Diatoms with symbiotic cyanobacteria can form extensive blooms supplying large amounts of nitrogen in the mixed layer of the ocean. Because of the O_2-sensitivity of the nitrogenase enzyme, N_2-fixing cyanobacteria segregate N_2 fixation from O_2 produced by photosynthesis (Zehr & Ward, 2002).

Besides nitrogen fixation, the only other major source of fully reduced nitrogen is ammonification (Jaffe, 1992). Ammonification is the breaking down of organic nitrogen compounds, such as amino acids or nucleotides, into ammonia or ammonium under both aerobic

and anaerobic conditions (Ehrlich, 1990). Heterotrophic bacteria are principally responsible for the decomposition of organic compounds from dead plant or animal matter as a carbon source, and leave ammonia or ammonium (at neutral pH ammonia exists as the ammonium ion). Under anoxic conditions, ammonia is relatively stable, and it becomes the predominate nitrogen in most anaerobic sediments. In soils, the majority of ammonia released by aerobic decomposition is rapidly recycled and converted to amino acids in plants and microorganisms. Ammonia can be also volatilized in soils. On a global basis, however, ammonia contributes only about 15% of nitrogen released to the atmosphere. The rest of the nitrogen released to the atmosphere is primarily in the form of N_2 or N_2O from denitrification (Madigan & Martinko, 2006).

Ammonia can be utilized as an energy source or nutrients for growth of microorganisms. Ammonia assimilation is the process by which ammonia or ammonium is taken up by an organism and converted to part of its biomass as organic nitrogen compounds. Organisms are also able to use NO_3^- or NO_2^- as nitrogen sources, although NH_3/NH_4^+ is preferred due to less energy requirements. NH_3 and NH_4^+ have already been reduced, whereas NO_3^- and NO_2^- require reductive assimilation (Jaffe, 1992). Nitrate assimilation process is initially catalyzed by assimilatory nitrate reductase. The nitrite is sequentially reduced to ammonia by assimilatory nitrite reductase (Zehr & Ward, 2002). The ammonia then is converted to glutamine to be incorporated into biomass (Kirby, 1981).

Nitrification is the aerobic oxidation of ammonia to nitrite then nitrate for energy production. There are several organisms that utilize nitrification as an energy source that are widespread in soil and water. The first step in the process (the oxidation of ammonia to nitrite) is principally done by autotrophic ammonia oxidizing bacteria (AOB), such as *Nitrosomonas*, and the second step (the oxidation of nitrite to nitrate) by mixotrophic nitrite oxidizing bacteria

(NOB), like *Nitrobacter*. AOB use ammonia monooxygenase to oxidize ammonia to hydroxylamine, an important intermediate in the nitrification process. Hydroxylamine is further oxidized to nitrite by hydroxylamine oxidoreductase. Nitrite oxidoreductase catalyzes the oxidation of nitrite to nitrate in NOB organisms (Madigan & Martinko, 2006).

Denitrification is a dissimilatory microbial redox process where nitrogen oxides (NO_3-, NO_2-) are reduced stepwise to gaseous end products (N_2O and N_2), which are concurrently released into the environment (Braker et al., 2001). Denitrification is considered to be the main way to remove nitrogen from the biosphere. The enzyme involved in the first step, dissimilative nitrate reductase, reduces nitrate to nitrite. Nitrite is reduced to nitric oxide (NO) by nitrite reductase. The reduction of nitric oxide to nitrous oxide is catalyzed by nitric oxide reductase, and the subsequent reduction of nitrous oxide is catalyzed by nitrous oxide reductase (Madigan & Martinko, 2006). The rate-limiting step in denitrification is nitrite reduction to nitric oxide (Santoro et al., 2006). Denitrification is the only process in which the major end product is removed from the internal biological nitrogen cycle. It is the principal means of balancing the input flux from biological nitrogen fixation (Jaffe, 1992). In agriculture it accounts for 20 to 30% of fertilizer losses, and in marine environments it accounts for up to 80% inorganic nitrogen inputs to coastal areas from terrestrial systems (Gruntzig et al., 2001).

Nitrate is reduced to NH_3 by another dissimilatory pathway, nitrate ammonification or dissimilatory nitrate reduction to ammonium (DNRA). DNRA may be in direct competition with denitrification for nitrate. The ratio between electron donors, usually a carbon source, and electron acceptors, such as nitrate, influences which pathway is followed (denitrification or nitrate ammonification) and which end products are produced (N_2 gas or ammonia). DNRA is favored when nitrate is limiting but electron donors are present in high numbers, but

denitrification is favored when electron donors are limiting but electron acceptors are present in high numbers (Kelso et al., 1997). Therefore, nitrate and nitrite reduction to ammonia dominate electron-rich environments where low concentrations of nitrate are available, such as human and animal gastro-intestinal tracts, polluted estuarine sediments, and environments saturated with sulfides (Mohan et al., 2004). Once nitrate is reduced to nitrite by dissimilatory nitrate reductase, nitrite is further reduced to ammonia in DNRA. The reduction of nitrite is catalyzed by the cytochrome *c* nitrite reductase, which is different from the enzyme used in denitrification (Simon, 2002). In nitrite ammonification, the gene responsible for cytochrome *c* nitrite reductase is NrfA (Simon, 2002). Ammonia produced by DNRA may be further used and recycled through ammonia assimilation or nitrification.

The global nitrogen cycle is characterized by the maintenance of a small pool of fixed or combined nitrogen in continuous exchange with the huge reservoir of atmospheric dinitrogen. Microbial nitrogen fixation is the most important natural process that converts atmospheric dinitrogen to fixed nitrogen (Thamdrup & Dalsgaard, 2002). The availability of fixed inorganic nitrogen (nitrate, nitrite, and ammonium) limits primary productivity in many oceanic regions. The conversion of nitrate to nitrogen gas through denitrification was believed to be the only important sink for fixed inorganic nitrogen in the ocean, until recently (Codispoti & Christensen, 1985; Thamdrup & Dalsgaard, 2002). The balance between these source and sink terms of fixed nitrogen are important regulators of ecosystem function and global biogeochemistry.

The New Paradigm of the Nitrogen Cycle: Anaerobic Ammonia Oxidation

Anaerobic ammonium oxidizing (anammox) bacteria were first predicted by the absence of NH_4^+ accumulation in active denitrification zones in the ocean. Oceanographers have known that far less ammonium accumulates in anoxic fjords and basins than would be expected from denitrification. In order to explain this shortfall, it was suggested that microorganisms are able to combine ammonium and nitrate to yield N_2 under anaerobic conditions (Kuypers et al., 2005). Two decades ago E.Z. Broda predicted the existence of chemolithoautotrophic anaerobic ammonia oxidizing bacteria (Broda, 1977). Chemolithoautotrophic organisms obtain the necessary carbon for metabolic processes from carbon dioxide in their environment and use inorganic compounds such as nitrogen, iron, or sulfur for the energy to power these processes (Madigan & Martinko, 2006). For a long time, the oxidation of ammonia was believed to be restricted to oxic environments (Jetten et al., 1999), although; anammox is actually energetically more favorable than oxic nitrification (Broda, 1977). It was on the basis of such thermodynamic calculations that Broda made his predictions that were confirmed only recently (Jetten et al., 2001). This process can explain ammonium deficiencies in anoxic waters and sediments, and it may contribute significantly to oceanic nitrogen budgets (Thamdrup & Dalsgaard, 2002).

Anammox oxidizes ammonium to N_2 while reducing nitrite as the terminal electron acceptor under strictly anoxic conditions (Figure 2) (Egli et al., 2001).

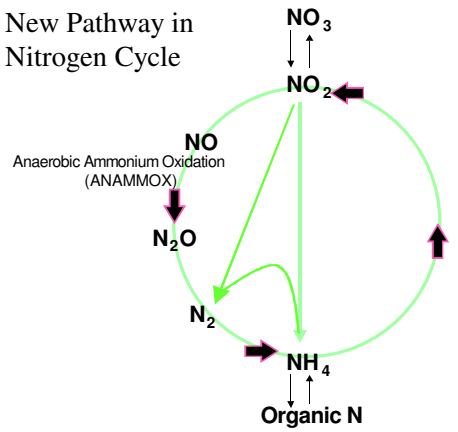

Figure 2. Diagram of the new microbial nitrogen cycle with the anammox loop

Anammox bacteria utilize nitrite and ammonium in a 1:1 ratio to form N_2 gas (Dalsgaard et al., 2003). Ammonium may be supplied from the water column by nitrate ammonification or through mineralization. Nitrate, and reduced nitrite, is produced as intermediates through the processes of nitrification, denitrification, and dissimilatory nitrate reduction. Oxygen completely inhibits anammox activity, but that the inhibitory effect of oxygen is indeed reversible once oxygen is removed (Jetten et al., 1999). Therefore, anammox is a new pathway in the biogeochemical nitrogen cycle. It converts nitrite directly to nitrogen gas without the release of N_2O gas (greenhouse gas), which is in denitrification. It also fully oxidizes ammonia to nitrogen gas anaerobically, whereas nitrification oxidizes ammonia to nitrate under oxic conditions.

Anammox in the Environment

Anammox bacteria was first discovered a decade ago in a pilot plant treating wastewater from a yeast-producing company in Delft, Netherlands when it was noted that ammonium disappeared from the reactor effluent at the expense of nitrate with a concomitant increase in dinitrogen gas production. After the first discovery nitrogen losses, which could only be explained by the anammox reaction, were reported in other wastewater treatment facilities including landfill leachate treatment plants in Germany, Switzerland, and England, as well as in semitechnical wastewater treatment plants in Germany, Belgium, Japan, Australia, and the United States (Schmid et al., 2005).

Nitrogen removal is an important aspect of wastewater treatment often accomplished by microbial processes combining nitrification and denitrification. These reactions have been known for a long time and have been successfully applied in most modern wastewater treatment

plants. The advantages of the anammox process over the traditional combination of nitrification and denitrification for wastewater treatment are lower oxygen demand and no requirement for external carbon sources (Egli et al., 2001). It is an excellent and low-cost alternative method for N-removal in wastewater treatment compared to the conventional nitrification/denitrification process (Kuai & Verstraete, 1998).

Anammox has also been discovered in natural ecosystems, such as the anoxic aquatic environments and anoxic sediments. In the highly stratified system of the Black Sea, anammox may consume more than 40% of fixed nitrogen that sinks down into the anoxic water column (Kuypers et al., 2003). Similar activity measurements were detected in the anoxic water column of Golfo Dulce, Costa Rica, where 19-35% of the total N_2 formation in the anoxic water column was attributed to the anammox reaction. Anammox activity also increased with increasing water depth where ammonium concentrations were highest (Dalsgaard et al., 2003). Recent studies also found anammox to be the main process responsible for nitrogen loss in the oxygen minimum zone (OMZ) waters of the Benguela upwelling system, one of the most productive regions of the world ocean (Kuypers et al., 2005).

Anammox activity has been detected in anoxic sediment as well; these activity measurements range from 0-67% of total N_2 removal by the anammox process. For example, in the Baltimore inner harbor anammox activity produced N_2 at a rate of only ~ 0.2 μM per gram of sediment per day (Tal et al., 2005). In continental shelf waters from East and West Greenland, the total N_2 production contributed to anammox ranged from 1-35% at eleven different sites (Rysgaard et al., 2004). On the other hand, anammox accounted for 67% of the total N_2 production in continental shelf sediments in the North Sea between Denmark and Norway, whereas denitrification was responsible for only 33% of N_2 formation. These studies show that

anammox plays an important and significant role in the biogeochemical nitrogen cycle, accounting for 19-40% of N_2 removal in the water column, and 1-67% of total N_2 removal in marine sediments (Dalsgaard et al., 2005).

Taxonomy of Anammox Bacteria

Anammox bacteria have a very slow growth rate (11 to 14 days), which hinders the isolation in pure culture from various environmental samples (Strous et al., 1999; Strous et al., 2002). However, anammox bacteria were highly enriched with a sequential batch reactor, which provided the sample to characterize their taxonomic linkages. The cells were purified from enrichment culture of the reactor using Percoll density centrifugation. The 16S rRNA genes in the cells were examined to obtain taxonomic identification. The first discovered anammox organism deeply branched in the *Planctomycetes* phylum and was named *Candidatus Brocadia anammoxidans* (Kuenen & Jetten, 2001). *Candidatus Brocadia fulgida*, another *Brocadia* bacteria was found from a wastewater treatment plant in Rotterdam, Netherlands, with 96% sequence similarity of 16S rRNA gene to *Ca. Brocadia anammoxidans* (Kartal et al., 2004). Through investigation of microbial community structure of a trickling filter biofilm with high anaerobic ammonium oxidation activity, *Candidatus Kuenenia stuttgartiensis* was identified (Schmid et al., 2000*). Candidatus Scalindua sorokinii* was found from 16S rRNA gene analysis of Black Sea water. (Kuypers et al., 2003). Two additional *Scalindua*-like bacteria (*Candidatus Scalindua brodae* and *Candidatus Scalindua wagneri*) were detected from 16S rRNA gene libraries generated from wastewater treatment plants (Schmid et al., 2003). After the discovery of *Ca*. *Scalindua brodae* and *Ca*. *Scalindua wagneri* in the wastewater treatment plant, they have

been found in marine environments as well, including the Black Sea and two estuaries in Denmark (Kuypers et al., 2003; Kirkpatrick et al., 2006; Risgaard-Petersen et al., 2004). *Ca. Scalindua brodae* was also discovered in a tropical freshwater lake (Schubert et al., 2006). Most recently Kartal et al. discovered a new propionate oxidizing species of anammox bacteria from a propionate degrading enrichment. This organism has been given the candidate name *Anammoxoglobus propionicus*. This bacteria has 91% 16S rRNA gene sequence similarity with other anammox bacteria. (Kartal et al., 2007). Phylogenetic analysis of these new anammox 16S rRNA sequences has shown that these bacteria form a monophyletic branch within the phylum *Planctomycetes* consisting of four distinct genera (*Ca. Scalindua*, *Ca. Brocadia*, *Ca. Kuenenia*, and *Ca. Anammoxoglobus*). These four genera have about 90% 16S rRNA sequence similarity to each other. *Ca. Brocadia*, and *Ca. Kuenenia* are associated with wastewater treatment plants and *Ca. Scalindua* is most commonly detected in natural systems (Schmid et al., 2005). Anammox bacteria have unique internal cellular structures as only found in other bacteria belonging to *Planctomycetes*. The major internal structure in anammox bacteria is a membrane-enclosed compartment called the anammoxosome. Unique lipids aggregate and form an unusually dense membrane structure that is highly resistant to diffusion, and this is where the anammox reaction takes place. The strong membrane of the anammoxosome likely protects the cell from toxic intermediates, such as hydrazine, produced during the anammox reaction (Madigan & Martinko, 2006). Although, anammox bacteria are similar in morphology to other *Planctomycetes* they differ on a genetic basis by organization of the rRNA operon. Bacteria belonging to the genera *Planctomycetes*, *Pirellula, Planctomyces*, and *Gemmata,* have unlinked 16S and 23S rRNA genes, but the genus *Isosphaera* and anammox bacteria have linked 16S and 23S genes. Anammox organisms' rRNA genes are linked with an intergenic spacer region of

only 450 bases (Schmid et al., 2001). The low 16S rRNA sequence similarity of anammox organisms to other genera of the *Planctomycetes* (below 80%) suggests that the anammox branch might be a second order within the *Planctomycetes* (Schmid et al., 2005).

Physiology and Biochemistry of Anammox Bacteria

The physiology of anammox bacteria has been extensively studied with an enriched culture of an anammox strain with 98.5-98.9% sequence similarity to *Kuenenia stuttgartiensis* and 90.9% sequence similarity to *Brocadia anammoxidans*. This enrichment is from a rotating disk contactor used to treat ammonium-rich leachate near Kolliken, Switzerland. Anammox activity has been observed only between pH 6.5 and 9, with an optimum at pH 8 and a temperature optimum at 37°C. High nitrite concentrations (~13.2 mM) did seem to inhibit anammox activity. Anammox activity was inhibited with oxygen concentrations as low as 0.25% (Egli et al., 2001). Anammox bacteria have a very high affinity for NH_4^+, which has been reported to be less than 5μmol L^{-1} (Strous et al., 1999). Another method, other than enrichment cultures, used to understand anammox physiology and biochemistry is environmental genomics.

Environmental genome of *K. stuttgartiensis* enrichment cultures has shown that nitric oxide (NO) is an important intermediate in anammox metabolism, and that anammox bacteria have a nitrite reductase gene similar in function to the *nir*S gene active in denitrification, reducing nitrite to NO. Hydrazine hydrolase produces hydrazine (N_2H_4) through the combination of NO and NH_4^+. Hydrazine is then oxidized to N_2 gas through a hydroxylamine-oxidoreductase-like protein. It was also proposed that anammox bacteria make use of the acetyl-coenzyme A (CoA) pathway for carbon fixation. The acetyl-CoA pathway depends on electrons

with a very low redox potential, which are usually derived from molecular hydrogen. Interestingly however, anammox bacteria derive their electrons from the anaerobic oxidation of nitrite to nitrate (Strous et al., 2006). Therefore, anammox has a very unique physiology in comparison with denitrifying and nitrifying bacteria. Anammox bacteria divide only once per two weeks at maximum speed, and produces nitric oxide and hydrazine (rocket fuel) as intermediates (Schalk et al., 2000). It is an autotrophic process requiring no external organic carbon source, which is very different from denitrifying bacteria. Denitrifying bacteria have a rapid doubling time (hours) (Rysgaard et al., 2004), and produce greenhouse gases as intermediates. Their heterotrophic lifestyle makes them dependent on organic carbon, and therefore the availability of organic matter may limit denitrifying bacteria in sediments underlying deeper waters (Rysgaard et al., 2004). Anammox bacteria oxidize NH_4^+ anaerobically, which is very different from AOB. AOB have low growth yields and produce hydroxylamine and nitrite as intermediates and employ the Calvin cycle for CO_2 fixation. Therefore, anammox bacteria have unique physiology and biochemistry for the oxidation of NH_4^+ and reduction of NO_2-, which differs from the traditional nitrification coupled to denitrification.

Methods Incorporated for the Study of Anaerobic Ammonium Oxidizing Bacteria

Cultivation of anammox is a challenging task since many cultivation techniques are not designed to deal with very slowly growing microorganisms, such as anammox bacteria. The doubling time of anammox bacteria has been reported to be from 11 to 30 days (Strous et al., 1998; Egli et al., 2001). A powerful tool that may be applied and optimized for the enrichment

and quantitative study of a very slow growing microbial community, such as anammox bacteria, is the sequencing batch reactor (SBR). Through the use of constant influent and effluent pumps and a gas buffer system, conditions always remain optimal for anammox bacterial growth. The SBR has proven to be advantageous for anammox enrichment because of the following strong points: efficient biomass retention, a homogeneous distribution of substrates allowing products and biomass to aggregate over the reactor, reliable operation for more than a year, and stable conditions under substrate-limiting conditions (Strous et al., 1998). Through the use of a 15-liter anaerobic SBR, which was fed with ammonia and nitrite mineral media, followed by Percoll density centrifugation, *Ca. Brocadia anammoxidans* was first purified to a density of 10^{10}- 10^{11} cells/ml (Strous et al., 1999; Kuenen & Jetten, 2001). By using the SBR, 74% of bacteria in the reactor enriched to anammox bacteria. This led to the determination of several important physiologies of anammox bacteria, such as biomass yield, the maximum specific ammonium consumption rate, and the maximum specific growth rate (Strous et al., 1998). Egli et al. (2001) enriched an anammox culture from a rotating disk contactor using a bench-top method. Anammox bacteria were enriched in 2.25-liter flasks with two liters of mineral medium. The media contained ammonium as the electron donor, nitrite as the electron acceptor, and bicarbonate as the carbon source. The bottles were incubated in an anaerobic chamber filled with N_2 and traces of H_2. This enrichment led to a relative population size of 88% anammox bacteria, which was confirmed by 16S rDNA sequencing and FISH analyses. However, anammox bacteria have not been isolated in pure culture.

Anammox bacteria and their activities without cultivation can be examined with the isotope pairing technique (IPT), fluorescent in situ hybridization (FISH), ladderane lipid detection, and 16S targeted polymerase chain reaction (PCR) studies. ^{15}N IPT is the most

common method to measure anammox activity in anoxic water and sediment samples. Either $^{15}NO_3$- or $^{15}NH_4^+$ is used as the nitrogen substrate during incubation. Denitrification combines two NO_3-, and anammox bacteria utilize NO_2- and NH_4^+ in a 1:1 ratio to form N_2 gas. For example, in an incubation with $^{15}NO_3$- and $^{14}NH_4^+$, denitrification would produce $^{30}N_2$ from the combination of two $^{15}NO_3$-. On the other hand, the anammox reaction would produce $^{29}N_2$ by combining each of the nitrogen sources. In an incubation with the treatment of $^{14}NO_3$- and $^{15}NH_4^+$, denitrification would produce $^{28}N_2$ by combining two $^{14}NO_3$-, but the anammox reaction would again produce $^{29}N_2$. Therefore, anaerobic incubation of water and sediment samples after the addition of $^{15}NH_4^+$ or $^{15}NO_3$- resulting in $^{29}N_2$ production in samples provides direct evidence for removal of fixed inorganic nitrogen by the anammox process (Kuypers et al., 2005).

Anammox bacteria can be detected by chemical analysis of cellular fatty acids and lipids since they have unique lipids, called ladderane lipids, and branched fatty acids, such as a variety of hopanoids (Schmid et al., 2005; Sinninghe Damste et al., 2004). To carry out lipid extractions from natural settings, large volumes of water must be filtered (Kuypers et al., 2003). If biomass is great enough, as is often the case in wastewater treatment plants, cell material may be obtained directly. Filters or cell material are then freeze-dried to isolate intact polar lipids (Boumann et al., 2006). Samples are then saponified to separate fatty acid and neutral lipid fragments. Total lipid extracts are then analyzed by gas chromatography- mass spectrometry for identification and quantification of ladderane lipids (Kuypers et al., 2003). Anammox bacteria are the first strict anaerobes that have been shown to biosynthesize these bacterial membrane rigidifiers (Schmid et al., 2005).

Molecular methods using FISH (fluorescent in situ hybridization) can be a standard method to detect anammox organisms. FISH delivers qualitative and quantitative data about the

bacterial population in a sample. Since anammox organisms are affiliated with the phylum *Planctomycetes*, a *Planctomycete* specific probe that also hybridizes with the 16S rRNA gene of anammox bacteria is good for initial detection. However, the probe not only hybridizes with 16S rRNA of anammox organisms but also any other bacteria belonging to *Planctomycetes*. A specific detection of anammox bacteria in environmental samples was initially performed in bioreactors and wastewater treatment plants. Most of these samples consisted of up to 50% of a single species of anammox bacteria (*Ca. Kuenenia stuttgartiensis* or *Ca. Brocadia anammoxidans*). Consequently, the majority of the initial probes designed for the detection of anammox bacteria are targeted to these two species of anammox. The recent discovery of members of the anammox genus, *Ca. Scalindua*, in a landfill leachate treatment plant in Pitsea, England, and in marine environments showed the probes constructed for identification of *Kuenenia* and *Brocadia* species were not sufficient to detect all anammox bacteria, and other probes were designed for detection of *Scalindua* species. The application of the probes specific for *Scalindua* species revealed that the plant in Pitsea is exceptional so far, since it is the only habitat that contains two different anammox species (*Kuenenia* and *Brocadia*) in almost equal amounts. Most importantly, the detection of *Scalindua* species in marine environments with FISH probes has shown the involvement of anammox bacteria in the global nitrogen cycle (Kuypers et al., 2005).

PCR detection of the16S rRNA gene is another alternative method to detect anammox organisms in the environment. This is especially useful when the cell counts are too low for FISH. A more directed PCR approach is commonly done with a *Planctomycete* specific forward primer (Pla46) and a general 16S rRNA gene-targeted reverse primer such as 1390R. Still, these primer pairs and subsequent cloning may not yield a quantitative representation of anammox

bacteria. Therefore, specific PCR for anammox 16S rRNA genes is required. For this reason, many anammox-specific FISH probes have been successfully applied as PCR primers. The combination of primers that seems to be the most effective are Pla46 as the forward primer with either the reverse primer Amx820 for *Brocadia*-like organisms, Sca1309 for *Scalindua*-like bacteria, or Amx368 for all known anammox bacteria. By applying the different sets of primers mentioned above, it is possible to distinguish between different groups of anammox organisms. However, PCR products should be further examined through sequencing analysis to confirm their phylogenetic affiliations (Schmid et al., 2005).

Molecular methods have limitations to detect diverse anammox bacteria, yet, FISH is often considered the gold standard for studying anammox bacteria. Although this is a very good method for detection, quantitation, and qualitative purposes, it does not allow further investigation into phylogenetic relationships between what is detected and known anammox sequences. FISH can also be rather time consuming and labor intensive as well. Dependent upon the stringency used, a single sequence mismatch can lead to weak hybridization, and often many probes are required to obtain a signal. From many PCR studies of the anammox 16S rRNA gene, anammox organisms are underrepresented in 16S rRNA gene clone libraries (Schmid et al., 2005). One reason for this is that presently used primer sets for the identification of anammox bacteria are inadequate leading to low PCR efficiency and a limited database of anammox bacterial sequences (Penton et al., 2006). Currently used primer sets for detecting anammox bacteria are not specific to anammox sequences only, but *Planctomycetes* and other groups closely related to *Planctomycetes* in general. In one study performed by Schubert et al. (2006), out of 400 clones sequenced from Lake Tanganyika, 49 were unique to *Planctomycetes,* but only one was closely related to anammox. The primers used for this study were the

Planctomycetales-specific Pla46F and the anammox-specific Amx368F paired with the universal 1392R primer. In another study carried out in Stuttgart, Germany at a semitechnical plant where the abundance of anammox organisms was 99% of the planctomycete population and over 40% of the bacterial population, only 9 out of 25 clones carried the anammox 16S rRNA genes with use of the Pla46F/1390R primer pair (Schmid et al., 2000). Another popular method used in anammox work is stable nitrogen isotope tracer techniques. This method has proved to be useful for elucidating differences in anammox and denitrification rates and understanding the key players involved in nitrogen removal. However, without proper molecular techniques to identify the individuals responsible for producing the N_2, a critical piece of the anammox puzzle will remain unsolved. Therefore, this study employs the combination of molecular techniques and IPT to overcome the disadvantages presented by these methods when used alone.

Study Site: The Cape Fear River Estuary

The Cape Fear River Estuary was chosen as the study site because it is a light-limited estuary where autotrophic cycling of nitrogen is minimal. Microbial processes dominate the cycling of nitrogen in the estuary. Marine and estuarine ecosystems in North Carolina are enduring increased nitrogen inputs as a result of large development of the North Carolina coastal zone. With increased nutrient loading, a secondary impact is affecting the Cape Fear River Estuary ecosystem as well, increased freshwater withdrawal from estuarine headwaters. Several bacteria mediated nitrogen cycling reactions can be altered by a change in salinity either through a shift in microbial communities, enzyme inhibition, or physico-chemical controls (Jones & Hood, 1980, Yoshie et al., 2004; Sorenson, 1987; Portnoy & Giblin, 1997, de Bie et al., 2001;

Stehr et al., 1995). The ecology of anammox bacteria in respect to salinity is unknown. To better understand the effects of nutrient loading and salinity change on the nitrogen cycle in the Cape Fear River Estuary, a key player in nitrogen removal was monitored, anammox bacteria. Anammox activity and diversity was measured at sampling sites of varying nitrate, ammonium, and salinity levels.

The previously existing PCR protocols for anammox identification was improved by designing a primer set specific to anammox bacteria only. In contrast with other *Planctomycetales* genera, anammox organisms possess linked 16S and 23S rRNA genes by an intergenic spacer region (ISR) of approximately 450 bp (Schmid et al., 2001). Therefore, by using a nested PCR approach with primers targeting each the 16S and 23S rRNA genes in the initial PCR, the detection of distantly related *Planctomycetes* that do not have linked 16S and 23S rRNA genes was eliminated. A method for measuring anammox and denitrification activity in established sediment incubations was developed as well. By monitoring the diversity and activity of anammox bacteria in relation to these environmental parameters, a clearer picture of anammox community dynamics may be provided. *The objective of this project is to examine anammox bacterial diversity, distribution, and activity in the Cape Fear River Estuary using molecular techniques and stable isotope techniques to determine the environmental factors influencing their community structure.*

Hypotheses:

H_{A1}: Diverse anammox bacteria will be present in the Cape Fear River since bacterial communities control nitrogen cycling in the estuary.

H_{A2}: Diversity of anammox bacteria will be positively correlated to levels of substrate (NH_4^+/NO_3) along the Cape Fear River.

H_{A3}: Distinct community structure of anammox bacteria will be present in different Cape Fear River sampling sites based on environmental parameters specific to that site. Therefore, anammox bacteria are niche specialists, and preferentially reside in well-adapted environments.

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CHAPTER 2. MOLECULAR DETECTION OF ANAMMOX BACTERIAL COMMUNITIES IN THE CAPE FEAR RIVER ESTUARY

INTRODUCTION

Anaerobic ammonium oxidation (anammox) is a new sink of fixed nitrogen, in which denitrification was formerly the only process. The anammox process, oxidizing ammonia (NH_4^+) while reducing nitrite (NO_2^-) under anaerobic conditions, was first predicted by Broda in 1977, as a more stoichometrically favorable process than aerobic ammonium oxidation (Broda, 1977). However, anammox was not confirmed until its discovery in a wastewater treatment plant in the Netherlands in 1995 (Mulder et al., 1995). Since then, it has been found to be a key player in anoxic ocean basins (Dalsgaard et al., 2003; Kuypers et al., 2003; Kuypers et al., 2005; Tal et al., 2005; Penton et al., 2006), estuaries (Risgaard-Petersen et al., 2004; Tal et al., 2005; Trimmer et al., 2003) freshwater systems (Schubert et al., 2006; Penton et al., 2006), and even Arctic sea ice (Rysgaard & Glud, 2004). Anammox significantly contributes to the global nitrogen cycle, accounting for up to 67% of anaerobic N_2 production in some areas (Thamdrup and Dalsgaard, 2002).

Anammox bacteria are extremely slow growing organisms with 11 to 14 days of generation time, which may have limited the evolution and diversity of anammox bacteria in nature (Schmid et al., 2005). The first anammox bacteria were cultivated as a stable consortium dominated with anammox bacteria from a wastewater treatment plant after substantial efforts (Strous et al., 1998). On the basis of 16S rRNA gene analysis, the enriched anammox bacteria were assigned to the *Planctomycetes* phylum with species name, *Candidatus Brocadia anammoxidans* (Kuenen & Jetten, 2001). More anammox bacteria were since discovered from wastewater treatment systems based on 16S rRNA gene analysis and named *Candidatus Kuenenia stuttgartiensis* as a new genus with 90% similarity with the *Brocadia* genus (Jetten et al., 2001). Kuypers et al. (2003) found the first marine anammox bacteria from the Black Sea,

the world's largest natural anoxic basin, and it was named *Candidatus Scalindua sorokinii*. Molecular analysis of various anaerobic environments detected *Candidatus Scalindua brodae, Candidatus Scalindua wagneri*, and *Candidatus Brocadia fulgida* (Schmid et al., 2003; Kartal et al., 2004). Kartal et al. (2007a) recently discovered a new anammox bacteria (*Candidatus Anammoxoglobus propionicus*) from a propionate degrading enrichment. Molecular detection of anammox bacteria from various ecosystems including wastewater treatments (Egli et al., 2001; Schmid et al., 2000; Schmid et al., 2001; Tal et al., 2006) marine sediments (Dalsgaard and Thamdrup, 2002; Engstrom et al., 2005; Freitag and Prosser, 2003; Meyer et al., 2005; Rich et al., 2007; Risgaard-Petersen et al., 2004; Rysgaard et al., 2004; Tal et al., 2005; Thamdrup and Dalsgaard, 2002; Trimmer et al., 2003), anoxic water basins (Dalsgaard et al., 2003; Kirkpatrick et al., 2006; Kuypers et al., 2003; Kuypers et al., 2005) and a freshwater lake (Schubert et al., 2006) showed three common features of anammox bacteria diversity. First, all four anammox bacteria are affiliated within 85% sequence similarity of 16S rRNA genes in the branch *Planctomycetes* except anammox bacteria found in Baltimore harbor sediments (Tal et al., 2005). Second, anammox bacteria especially assigned to the genera *Brocadia* and *Kuenenia* have specific niches since they were only found in engineered systems such as wastewater treatments, however; *Scalindua* like organisms were mostly present in natural ecosystems from freshwater lakes to anoxic marine environments. Third, only one phylogenetic group of anammox dominated in most of the examined ecosystems. Therefore, anammox bacteria diversity and distribution could be strongly related to various environmental factors, including salinity, ammonium and nitrate concentrations, and sediment mineralization. Further studies of anammox bacteria should be carried out to determine environmental and physiological factors that control abundance and activity of anammox bacteria in the environment.

The Cape Fear River is an excellent study site for the bacteria-mediated nitrogen cycling processes because it is a light limited system where autotrophic nitrogen cycling is minimal. Bacteria dominate nitrogen cycling in this estuary, and therefore; it was chosen as the study site to monitor one of the key players in N_2 production, anammox bacteria. Therefore, diversity and distribution of anammox bacteria in the Cape Fear River Estuary were examined with molecular detection methods and further correlation between community structure and environmental parameters were investigated to understand niche characteristics of anammox bacteria.

MATERIALS & METHODS

Sediment Collection

Sediment was collected with a petite Ponar grab sampler from three sampling sites in the Cape Fear River Estuary (Wilmington, North Carolina) in January, May, September, and November 2006. Water depth of each sample was approximately 1m. A subsample of the top 3 cm of sediment was collected and stored at -80°C until examination. These sites are of varying nitrate and salinity gradients (Figure 3 and Table 1).

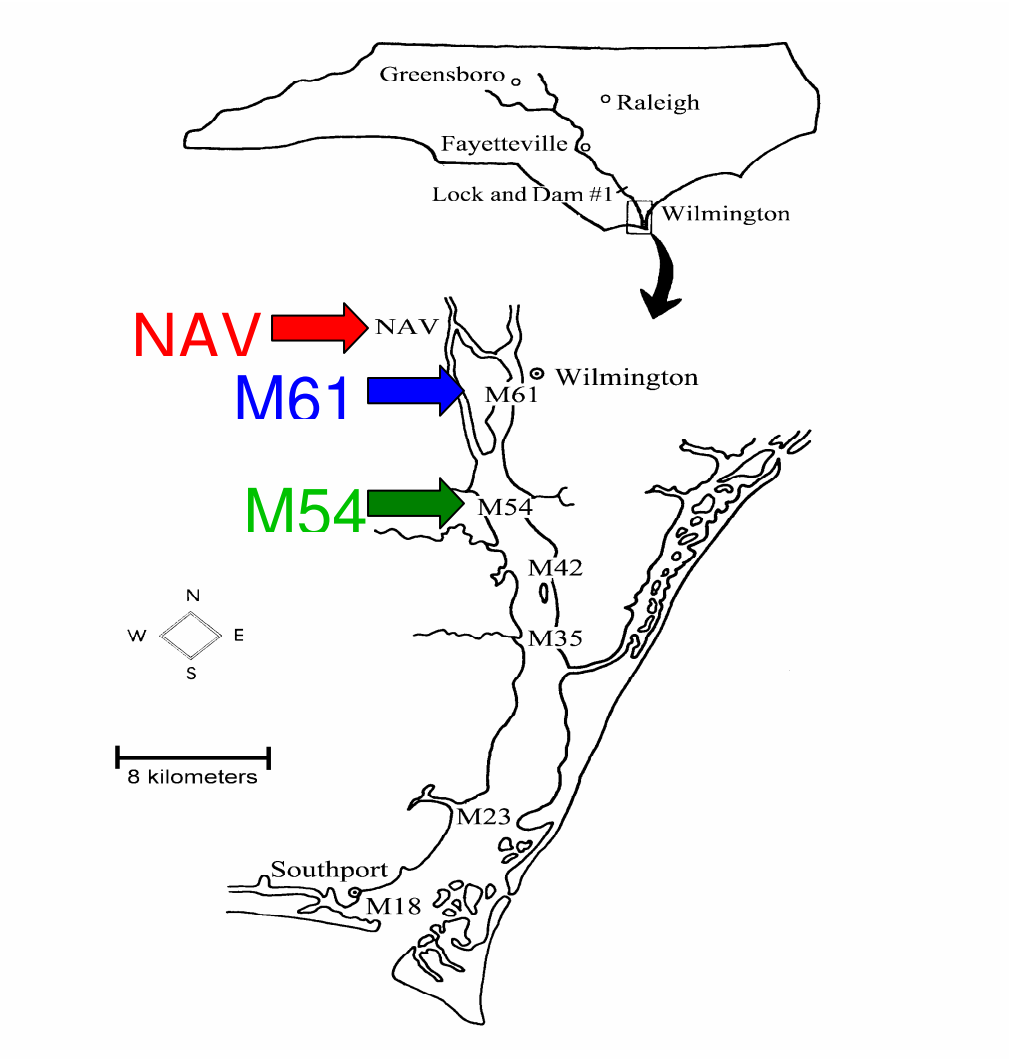

Figure 3. Map of the sampling sites in the Cape Fear River Estuary

Table 1. Environmental parameters and diversity estimates for sampling sites

Sampling Station	Mean Annual Salinity (ppt)	Mean Annual Ammonium (μM)	Mean Annual Nitrate (μM)	Chao 1 Estimate	Shannon Index
Cape Fear River Estuary, NC					
NAV (Upper Estuary)	0.9 ± 1.4	4 ± 2	6.9 ± 3.3	1	0
M61 (Mid Estuary)	6 ± 4.8	4.1 ± 1.6	5.7 ± 2.5	2	1.02818
M54 (Lower Estuary)	7.4 ± 5.1	5.2 ± 3.3	5.5 ± 2.6	6.333333	1.56437

Table 2. Salinity (ppt) for sampling sites for each sampling time and the two preceding months

(Sampling months are in bold font.)

Sampling Station	Nov. 2005	Dec. 2005	**Jan. 2006**	Mar. 2006	Apr. 2006	**May 2006**	July 2006	Aug. 2006	**Sept. 2006**	Oct. 2006	**Nov. 2006**
NAV	1.4	0.1	0.1	0.1	2.1	0.1	0.1	1.5	0	0.1	1.4
M61	9.7	2.1	0.2	9.9	12.1	6.7	0.9	10.1	0	10.5	3.2
M54	11.3	2.8	4.2	11	13.2	7.5	1.6	12.3	0.4	11.1	9.9

Table 3. Nitrate (μM) for sampling sites for each sampling time and the two preceding months

(Sampling months are in bold font.)

Sampling Station	Nov. 2005	Dec. 2005	**Jan. 2006**	Mar. 2006	Apr. 2006	**May 2006**	July 2006	Aug. 2006	**Sept. 2006**	Oct. 2006	**Nov. 2006**
NAV	5.8	2.2	4.4	6.7	6.6	4.2	6.6	4.3	1.3	5.2	5.5
M61	2.7	7.2	0.4	4.4	5	4.1	5.6	4.5	0.8	3.9	3.3
M54	1.1	8.5	3.5	3.6	4.1	1.9	5.6	4.3	1	3.2	3

Table 4. Ammonium (μM) for sampling sites for each sampling time and the two preceding months

(Sampling months are in bold font.)

Sampling Station	Nov. 2005	Dec. 2005	**Jan. 2006**	Mar. 2006	Apr. 2006	**May 2006**	July 2006	Aug. 2006	**Sept. 2006**	Oct. 2006	**Nov. 2006**
NAV	5	3.8	3.8	5.5	7.2	6.1	2.7	1.1	1.6	3.3	3.8
M61	6.1	5.5	3.8	6.1	5.5	6.1	3.3	1.1	2.2	4.4	3.8
M54	8.8	6.1	5	6.5	9.4	6.6	2.7	1.1	2.7	3.8	3.3

All three of the sites in the Cape Fear River Estuary are monitored through the Lower Cape Fear River Monitoring Program, and therefore data such as salinity, nitrate and nitrite concentrations, and ammonium concentrations are measured and updated regularly on the Lower Cape Fear River Monitoring Program's website (http://www.uncwil.edu/cmsr/aquaticecology/lcfrp).

Extraction of DNA and PCR amplification

All environmental DNA was extracted from sediment samples using the MoBio Power Soil DNA Kit, following the manufacturer's instructions (MoBio Laboratories, Inc., Carlsbad, California). To detect anammox bacteria in the sediments two different PCR protocols were used. The first method was referred to as the Amx Pla PCR. First, amplification of *Planctomycetales*-specific 16S rRNA genes was done using the Pla46F primer and the 1390R universal bacterial primer. The nested anammox-specific 16S PCR was performed using the Pla46F primer and the AMX368R primer with 1µl of PCR product from the previous reaction as template. Each PCR (25 µl) contained 2.5 µl 10X PCR buffer (500 mM KCl, 200 mM Tris-HCL [pH 8.4], 2.5 mM $MgCl_2$ (25mM), 1 U of *Taq* (Promega, Madision, Wisconsin), 0.8 mM of each deoxynucleotide, 400 pmol of each primer, and 1 to 100 ng of DNA as template. The reaction cycle parameters of the first PCR included an initial denaturation step of 4 min at 94°C, followed by forty cycles of amplification; each cycle consisted of denaturation at 94°C for 45 s, primer annealing at 59°C for 50 s, and primer extension at 72°C for 3 min. The reaction cycle parameters of the second PCR were the same, except the second cycle's primer extension step was 72°C for 1 min. The size of the PCR product was determined by using gel electrophoresis

with a 1% (wt/vol) agarose gel and 1X Sigma TAE (Sigma-Aldrich Co., St. Louis, Missouri). Because this approach was not specific to anammox bacteria only, we developed another PCR program that amplified anammox bacteria sequences only, the anammox specific PCR (Figure 4, Table 5).

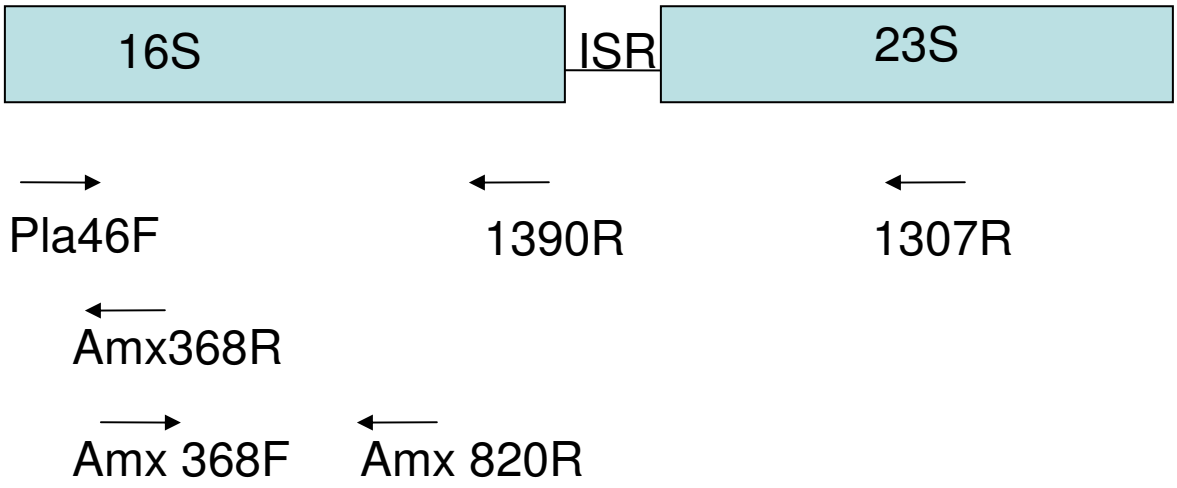

Figure 4. Schematic of primers used in Amx Pla and anammox specific PCR protocols

Table 5. PCR primers for anammox detection

Primer Name	Target gene	Orientation	Sequence (5' to 3')	Reference
Pla46F	Planctomycetes 16S	Forward	GACTTGCATGCCTAATCC	Neef et al., 1998
1390R	Eubacteria 16S	Reverse	GACGGGCGGTGTGTACAA	Olsen et al., 1986
Amx368R	Anammox 16S	Reverse	CCTTTCGGGCATTGCGAA	Schmid et al., 2005
1307R	Eubacteria 23S	Reverse	CGACAAGGAATTTCGCTAC	Ludwig et al., 1992
Amx368F	Anammox 16S	Forward	TTCGCAATGCCCGAAAGG	Schmid et al., 2005
Amx820R	Anammox 16S	Reverse	AAAACCCCTCTACTTAGTGCCC	Schmid et al., 2005

The initial PCR amplification for the anammox-specific PCR was performed with the Pla46F primer (specific to *Planctomycete* 16S rDNA) and the 1307R primer (specific to Eubacteria 23S rDNA) in a 25 µl volume containing PCR buffer (500 mM KCl, 200mM Tris-HCl [pH 8.4], 0.15 mM $MgCl_2$, 20µM of each deoxynucleotide, 1µM of each primer, 1 U *Taq* polymerase and 1 µl of DNA as template (10 to 100 ng). The PCR cycle was started with a 30 s denaturation step at 98°C, followed by 30 cycles of denaturation for 45 s at 98°C and primer annealing for 50 s at 59°C, and concluded by a 3-min extension step at 72°C. The nested PCR was performed with the anammox specific primers, Amx368F and Amx820R. The PCR was started with a 4-min denaturation step at 94°C, followed by 30 cycles of denaturation for 45 s at 94°C and primer annealing for 50 s at 59°C, and concluded by a 1-min extension step at 72°C. The reaction cycle parameters of the second PCR were the same, except the second cycle's primer extension step was 72°C for 1 min. The amplified products were examined in 1.0% agarose gels by electrophoresis and purified using the Eppendorf Perfect Gel Clean-Up Kit, following the manufacturer's instructions (Eppendorf, Brinkmann Instruments Inc., USA). PCR amplification by this anammox specific PCR was followed by cloning and sequencing.

Cloning, Sequencing, and Phylogenetic Analysis

Purified DNA fragments from January samples were introduced into a pCR2A vector and transformed into *Escherichia coli* by using a TOPO TA cloning kit, as instructed by the manufacturer (Invitrogen, Carlsbad, California). The transformed cells were plated on Luria agar plates containing 50 µg/ml of kanamycin. Clones with the inserts were sequenced using BigDye Terminator chemisty (Applied Biosystems, Foster City, Calif.) and a model 3100

automated sequencer (Applied Biosystems). DNA sequences were examined and edited using DNASTAR Lasergene SeqMan Program (DNASTAR, Inc., Madison, Wisconsin). NCBI BLAST (http://www.ncbi.nih.gov) was used to find the most closely related 16S rRNA gene sequences in the public databases. The partial 16S rRNA gene sequences were aligned using ClustalW (www.ebi.ac.uk/clustalw/). Neighbor-joining phylogenetic trees with bootstrapping were produced using MEGA, version 3.1 (Kumar et al., 2004).

Terminal Restriction Fragment Length Polymorphism (T-RFLP)

For all T-RFLP analysis sediment DNA was quantified with the Quant-iT™ PicoGreen® dsDNA Assay Kit, according to the manufacturer's protocol (Molecular Probes Invitrogen, Eugene, OR). The anammox specific PCR approach was used with 6' FAM-fluorescent labeled AMX368F primer. PCR products were gel purified with the Q-Bio Gene Geneclean® Turbo Kit, following the manufacturer's instructions (Q-Bio Gene, Irvine, CA), and used as template for T-RFLP analysis. Purified PCR products were digested with the *Alu*I restriction enzyme at 37°C overnight. *In silico* digestion determined that the *Alu*I restriction enzyme separated anammox bacterial sequences by genera as the restriction enzyme cut site (AG/CT) was found at different locations in 16S rRNA genes in each genera (Kuenenia, 269 bp; Scalindua, 60 bp; Brocadia, 284 bp & 398 bp). The digestion reaction mixture (35µl) contained 20 ng of the purified PCR product and 10 U of *Alu*I. Digested PCR products were precipitated with 75% isoproponol and resuspended in 10µl of Hi-Di formamide with GeneScan ROX 500 size standards. Samples were denatured at 95°C for 1 minute, followed by rapid chilling on ice. The

lengths of T-RFs were automatically determined by comparison with the internal size standards by using the GeneScan software, version 3.1.

Statistical Analysis

The DOTUR (Distance-Based OTU and Richness) program was employed to compare diversity for each annamox 16S rRNA sequence from each sampling site (Schloss & Handelsman, 2005). The number of OTUs was defined by a 1% difference in nucleotide sequences, as determined by using the furthest neighbor algorithm in DOTUR. DOTUR was also used to generate diversity analyses such as Chao 1 and Shannon index numbers for each sampling site (Table 1). MEGA 3.1 (Molecular Evolutionary Genetics Analysis) was also used to make pairwise comparisons within groups of anammox 16S rRNA genes.

RESULTS & DISCUSSION

Planctomycetes and Anammox Identification in the Cape Fear River Estuary

Anammox bacterial communities in two different sites (M54 and NAV) were examined with DNA extracted from the sediment collected in January 2006. The Pla Amx PCR was initially used. This is a nested PCR program that employs the use of the Pla46F/1390R primer set for the initial PCR, and the Pla46F/368R primer set for the second PCR (Table 5, Figure 4). This yields a PCR product of approximately 325 bp. Positive PCR products were cloned, and forty clones from each site were sequenced. Sequencing analysis revealed 16S rRNA gene clones with high sequence similarity to either *Planctomycetes* or known anammox bacteria (Figure 5).

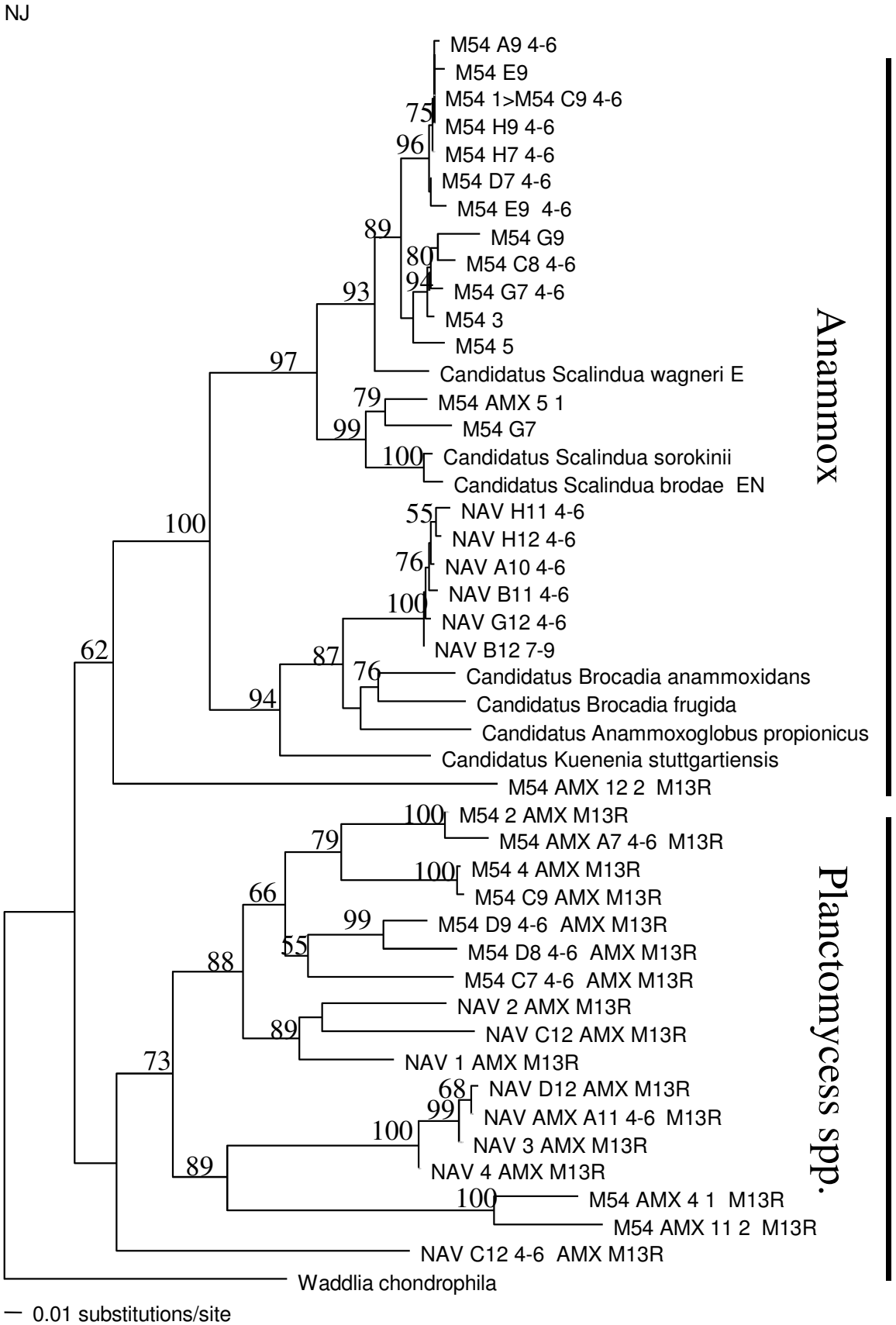

Figure 5. Phylogenetic tree of anammox bacterial and *Planctomycetes* 16S rRNA genes detected from January samples generated through Pla Amx PCR method

In site NAV, the uppermost sampling site in the Cape Fear River Estuary, 14 out of 40 clones sequenced were identified as *Planctomycetes* and 6 of these clones were identified as anammox bacteria. From site M54 in the middle of the Cape Fear River Estuary 25 out of 40 clones were identified as *Planctomycetes* and 14 of these clones were identified as anammox bacteria. Phylogenetic analysis of the detected anammox sequences showed that all 6 of the anammox sequences detected from site NAV grouped with *Brocadia* organisms, and all 14 of the anammox sequences detected from site M54 grouped with *Scalindua* organisms (Figure 5).

Using the Pla Amx PCR method, 20 out of 80 clones sequenced from January 2006 samples from sites M54 and NAV were identified as anammox 16S rRNA gene sequences through sequence analysis. However, 39 out of 80 clones sequenced were determined to be *Planctomycetes spp.* 16S rRNA gene sequences (Figure 5). Thus, the Pla Amx PCR is not very specific for the detection of anammox organisms. Further phylogenetic studies, such as tree building with known anammox organisms' 16S rRNA sequences and bootstrap analysis, must be carried out to determine whether the amplified 16S rRNA genes are in fact assigned to anammox bacteria. More *Planctomycete* organisms distantly related to anammox organisms, approximately 49%, were amplified through PCR, then anammox organisms themselves, only 25%. Therefore, by designing a primer set specific to anammox organisms only, the detection method was optimized to be more efficient for anammox study (Figure 4).

Specific Detection of Anammox Bacteria in the Cape Fear River Estuary

In order to more specifically detect anammox organisms, primers were targeted to sites on both the 16S and 23S rRNA genes in an initial PCR by using the Pla46F primer specific to the *Planctomycetes* 16S rRNA gene and the 1307R primer specific to Eubacteria 23S rRNA genes. Other *Planctomycete* organisms, members of the genera *Planctomyces, Pirellula*, and *Gemmata*, have genomically separated rRNA operons. Anammox organisms have linked 16S and 23S rRNA genes with 450 bp of the intergenic spacer region (ISR) (Figure 4) (Schmid et al., 2005). By doing this initial PCR, the detection of other *Planctomycete* organisms was eliminated because the large space between their 16S rRNA genes and 23S rRNA made it impossible for their sequences to be amplified. Only organisms with linked 16S and 23S rRNA genes (i.e. anammox) were amplified in the first PCR. In the nested PCR, the Amx368F primer and the Amx 820R primer, both specific to anammox sequences, were used (Table 5, Figure 4). This PCR method yielded a product of approximately 450 bp. The new detection method was very efficient, yielding 100% anammox sequence detection (Figure 6).

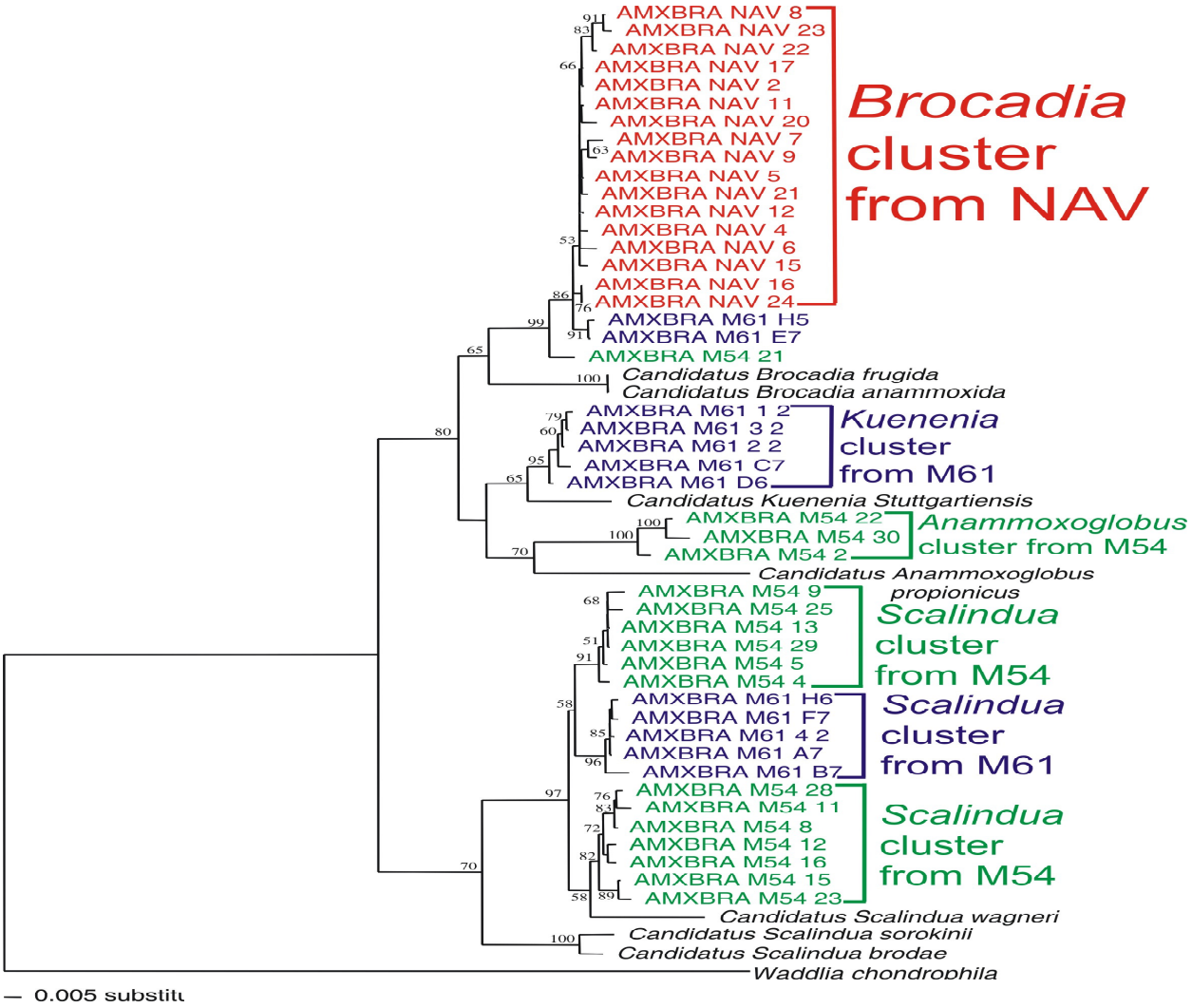

Figure 6. Phylogenetic tree of anammox bacterial 16S rRNA genes detected from January samples generated by anammox specific PCR protocol

With the anammox specific PCR, all clones sequenced from NAV were identified as *Brocadia* organisms with 99.4% sequence similarity to each other and 91% similarity to the sequences of the two known *Brocadia* organisms, *Candidatus Brocadia anammoxidans* and *Candidatus Brocadia fulgida*. Sequences from site M61 were closely related to three different anammox genera: *Brocadia, Scalindua*, and *Kuenenia*. The five sequences in the *Kuenenia* cluster from site M61 were 93.7% similarity to each other and 93.7% similarity to *Candidatus Kuenenia sorokinii*. The five sequences in the *Scalindua* cluster from site M61 had 99.5% sequence similarity to one another, and 89.2% similarity to *Candidatus Scalindua sorokinii, Candidatus Scalindua brodae*, and *Candidatus Scalindua wagneri*. Similar to the Amx Pla PCR protocol, when using the anammox specific PCR protocol to examine site M54, the majority of anammox organisms detected were most closely related to *Scalindua* organisms, but a few *Kuenenia* and *Anammoxoglobus propionicus* sequences were detected as well (Figure 6). Three sequences detected in site M54 had 98.7% sequence similarity to one another and 91.2% similarity to *Candidatus Anammoxoglobus propionicus.* Upon designing the anammox specific primers, this was the first time that any *Kuenenia* and *Anammoxoglobus propionicus* organisms were detected in any of our samples collected from the Cape Fear River Estuary. All of the sequences within the same cluster were more than 90% similar to one another, and all the clusters composed of similar species from different sites were more than 90% similar to each other and their reference sequences as well. The exception to this is the three *Scalindua* clusters, two from site M54 and one from site M61, and the *Anammoxoglobus propionicus* sequences. These clusters had lower than 90% sequence similarity to the *Scalindua* reference sequences. The uppermost *Scalindua* cluster on the tree from site M54 had 89.4% similarity to the *Scalindua* reference sequences. The *Scalindua* cluster from site M61 had 89.2% similarity to the *Scalindua*

reference sequences, and the other cluster from site M54 had 89.2% similarity to the *Scalindua* reference sequences as well. The level of anammox diversity at each sampling site in January was obtained through incorporation of rarefaction analysis (Figure 7). The operational taxonomic unit (OTU) determination was based on 1% sequence variation. NAV had the least anammox diversity of the three sites, because all of the organisms identified here were *Brocadia* organisms. Therefore, only one OUT was identified at site NAV. Site M61 had 3 different OTU's, and site M54 had the most anammox diversity with 6 different OTU's. This is depicted in the rarefaction analysis (Figure 7) and also indicated by the Chao 1 estimate and Shannon index numbers, which measure species species richness and eveness (Table 1). The rarefaction curve for site M54 did not reach saturation of OTU numbers, as did the curves for sites M61 and NAV. However, the Chao 1 estimator calculated at least 6 different species of anammox bacteria in site M54 based on 1% sequence variation.

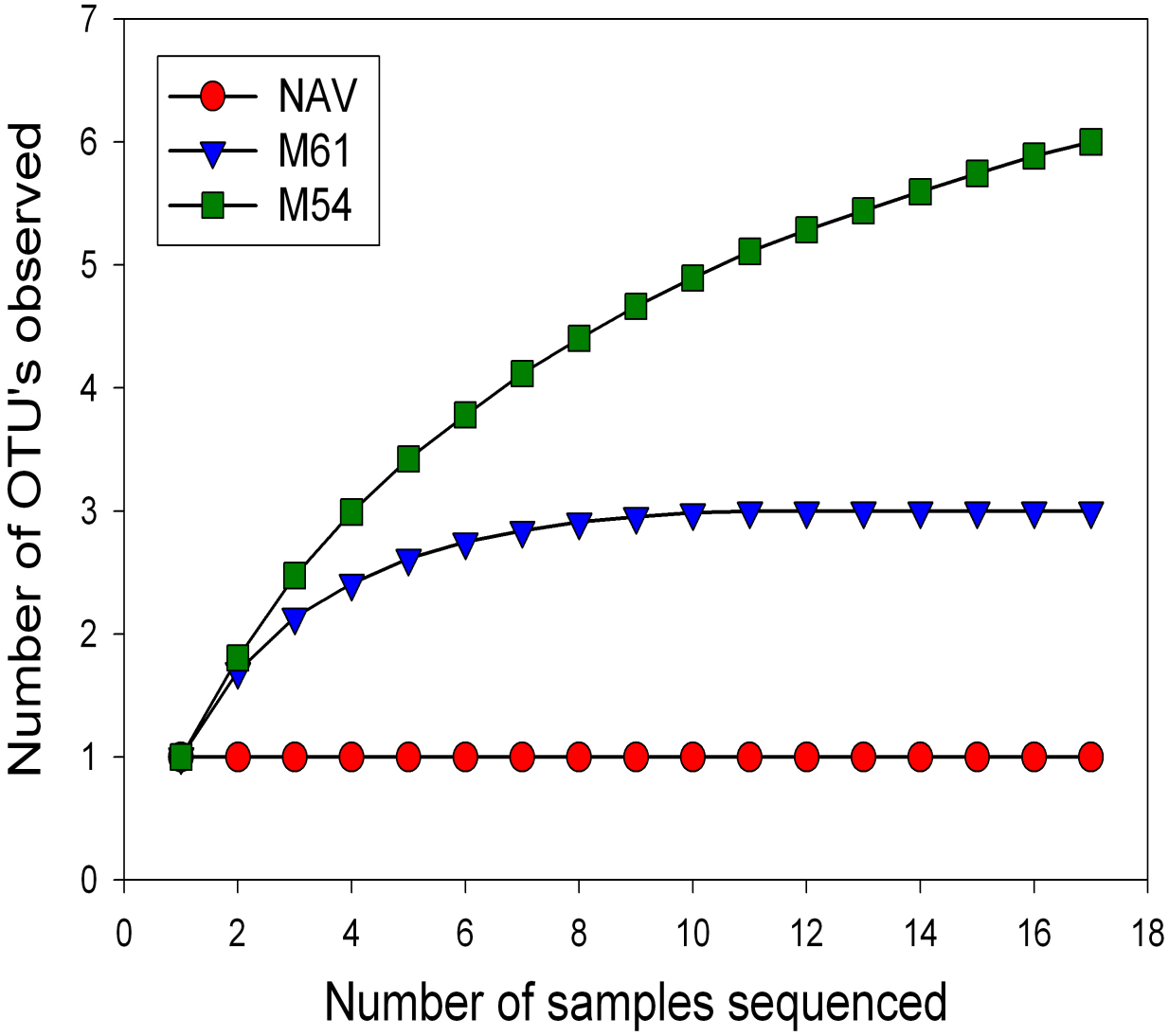

Figure 7. Rarefaction analysis of anammox 16S rRNA genes detected from January samples collected from each site

Seasonal Variation Detected through T-RFLP Analysis

Anammox community structure was further monitored in sediment samples collected in January, May, September, and November for each sampling site through T-RFLP analysis. For all sampling times at site NAV, the anammox community seemed to be monophyletic, with one genera dominating. *Brocadia* peaks (284 bp & 398 bp) only were detected at site NAV (Figure 8). There was a shift in *Brocadia* community structure in this site, however. The *Brocadia* community represented by the peak at 284 bp dominated during January, May, and September sampling dates. In November though, this *Brocadia* community declined drastically and another group of *Brocadia* organisms increased, the community represented by the peak at 398 bp. The environmental parameters of nitrate and ammonium vary little over these time periods at site NAV. However, the salinity in November reached 1.4 ppt while salinity in January, May, and September was 0 to 0.1 ppt (Table 2). Although, this is still essentially freshwater, perhaps the *Brocadia* community represented by the peak at 284 bp has a very narrow range of salinity tolerance. The only time this *Brocadia* peak at 284 bp appears again in any of the samples is in the M61 September sample. The salinity for this month at site M61 was also 0 ppt (Table 2).

Figure 8. T-RFLP analysis of anammox bacterial 16S rRNA genes for site NAV

Scalindua organisms only were detected at site M61 in January and May, represented by the peaks at 60 bp, while mixed anammox communities were present in September and November. Three different anammox genera (*Scalindua, Kuenenia*, and *Brocadia*) were detected in September. This sample had more anammox communities present than all the others based on T-RFLP analysis. In November, *Scalindua* and *Brocadia* organisms were detected. Scalindua organisms are present in every sampling date at site M61. These organisms also dominate at every sampling date, except in September when *Scalindua* and *Kuenenia* organisms are present in approximately equal levels, represented by the peaks at 60 and 269 bp, respectively. This is the only time at site M61 that *Kuenenia* organisms and the *Brocadia* community represented by the peak at 284 bp was detected (Figure 9). As with site NAV, the nitrate and ammonium levels for these different sampling dates vary little. Salinity at site M61 for September was 0 ppt (Table 2). Perhaps, *Kuenenia* is also a freshwater adapted organism. The resolution for anammox detection is much higher through sequence analysis than T-RFLP analysis. Five *Kuenenia* sequences were detected at site M61 during the January sample, as shown by the phylogenetic tree in Figure 6. However, no detectable peak with 269 bp size, representing *Kuenenia* organisms, was identified by T-RFLP for the January sample.

Figure 9. T-RFLP analysis of anammox bacterial 16S rRNA genes for site M61

For all sampling times, *Scalindua* organisms dominated at site M54. Small *Brocadia* peaks were detected in May, September, and November, and a small *Kuenenia* peak was present in September as well. Similar to the M61 September sample, the September sample at site M54 also had three different genera present. At site M61, two *Brocadia* communites (284 bp & 398 bp) were identified, but in site M54, only the *Brocadia* community represented by the peak at 398 bp was detected (Figure 10).

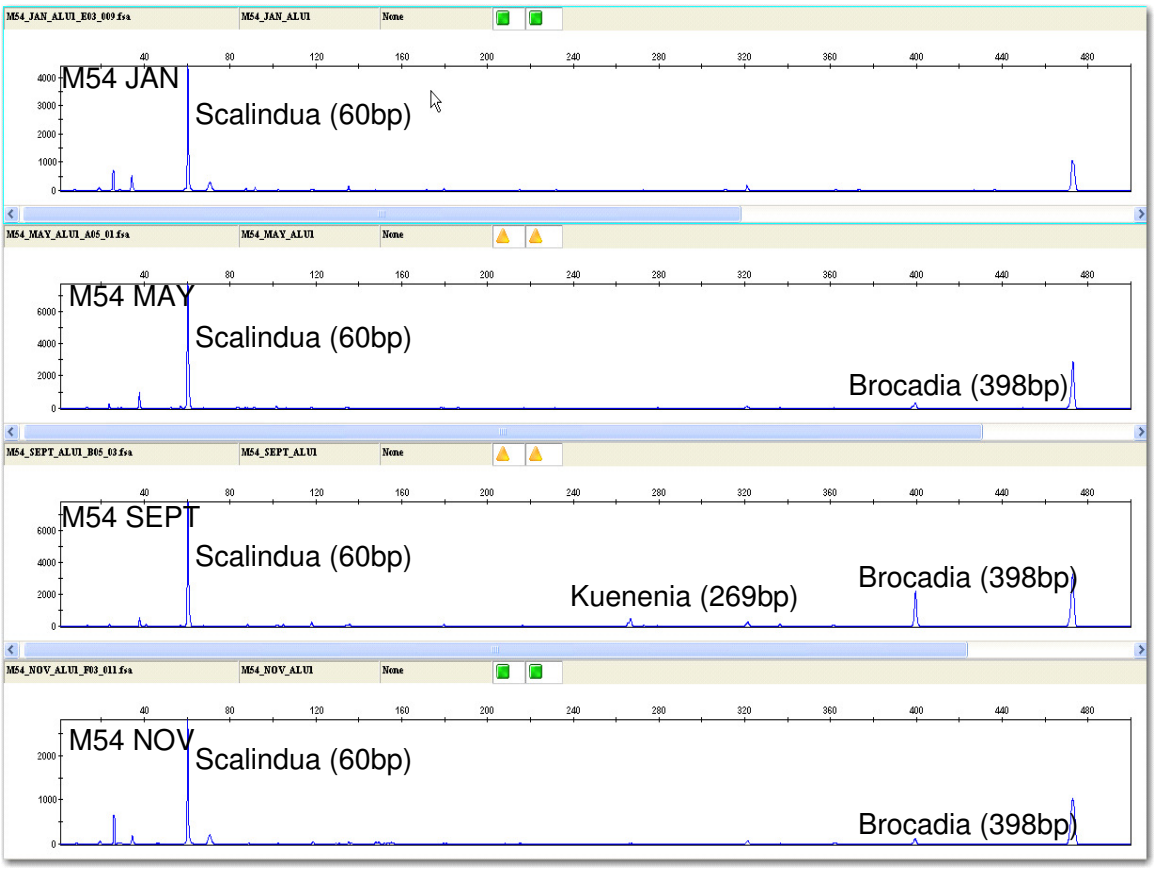

Figure 10. T-RFLP analysis of anammox bacterial 16S rRNA genes for site M54

At site M54, September had the lowest salinity (0.4 ppt) of the whole year (Table 2). This decrease in salinity correlates the appearance of the *Kuenenia* peak and the highest *Brocadia* peak at site M54, compared to May and November (Figure 10). The spring and fall seasons in Wilmington, NC, seem to correlate with dry and wet seasons, respectively. Rainfall was much higher in the fall than the spring (http://www.ncdc.noaa.gov/oa/ncdc.html), and therefore; the salinity levels in the Cape Fear River Estuary may have been lowered because of dilution by rainfall. This increase in rainfall in the fall season may have permitted *Brocadia* species to persist in site M61 and *Brocadia* and *Kuenenia* species to persist in site M54. Rainfall may also have served as a transport mechanism for these bacteria communities as well. With an increase in the standard deviation from the annual salinity levels comes an increase in diversity (Figure 14). This could well be the case that is represented by the increase in diversity between the spring and fall samples.

Comparison of Anammox Communities with Environmental Parameters

The difference of anammox communities in this study seemed to be related to the environmental parameters of salinity and ammonium, as shown by the increase in the Chao 1 estimate and the rarefaction analysis (Table 1). This analysis was performed on anammox sequences obtained from the anammox specific PCR on January samples (Figure 7). With increasing salinity and ammonium levels, the Chao 1 estimate number and curves on the rarefaction analysis increase. To further analyze possible relationships of anammox diversity with the environmental parameters of salinity, ammonium, and nitrate, anammox diversity, represented as OTU's, was plotted against the measured salinity, ammonium, and nitrate levels. Two time periods were taken into account for this analysis, the mean environmental parameters for the month the sample was collected and the two preceding months (Table 6), and the annual mean environmental parameters for each sampling site (Table 1). Under optimal conditions, the doubling time of anammox bacteria has been reported to be from 11 to 30 days (Strous et al., 1998; Egli et al., 2001). The doubling time in natural systems, such as the Cape Fear River Estuary, is unknown. However, because of the slow doubling time of anammox bacteria, the environmental parameters of each sampling month as well as the two previous months were considered in determining the effect of environmental conditions on anammox metabolism and growth. Because samples were collected in January, the graph below is based on the mean environmental parameters of November, December, and January (Table 6, Figure 11).

Table 6. Mean environmental parameters and fluctuation for January sampling

Sampling Station	Salinity (ppt)	Ammonium (µM)	Nitrate (µM)
NAV	0.5 ± 0.75	4.2 ± 0.69	4.13 ± 1.81
M61	4 ± 5.02	5.13 ± 1.19	3.43 ± 3.45
M54	6.1 ± 4.55	6.63 ± 1.95	4.36 ± 3.77

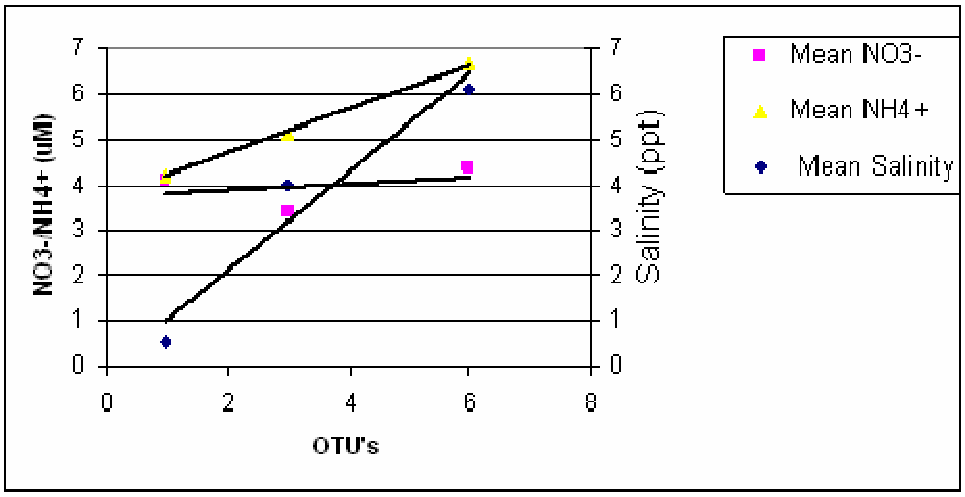

Figure 11. Comparison between mean environmental parameters for three months and anammox bacterial diversity at each sampling site

Salinity and bacterial diversity were positively correlated as shown in Figure 11. In addition, the Chao I estimate and Shannon index showed the increase of the calculated estimates by site as salinity increases. However, this relationship is not significant with a p-value of 0.16. There was no correlation between an increase in nitrate levels and anammox bacterial diversity. Because ammonia is rarely limiting in sediments, it is often not considered to be an important factor controlling anammox (Risgaard-Petersen et al., 2003). However, anammox bacterial diversity and ammonium levels increased together in this study, as shown by the positive correlation in Figure 11. This relationship was highly significant with a p-value of 0.01. These relationships were also observed on a broader time scale by considering the annual mean.

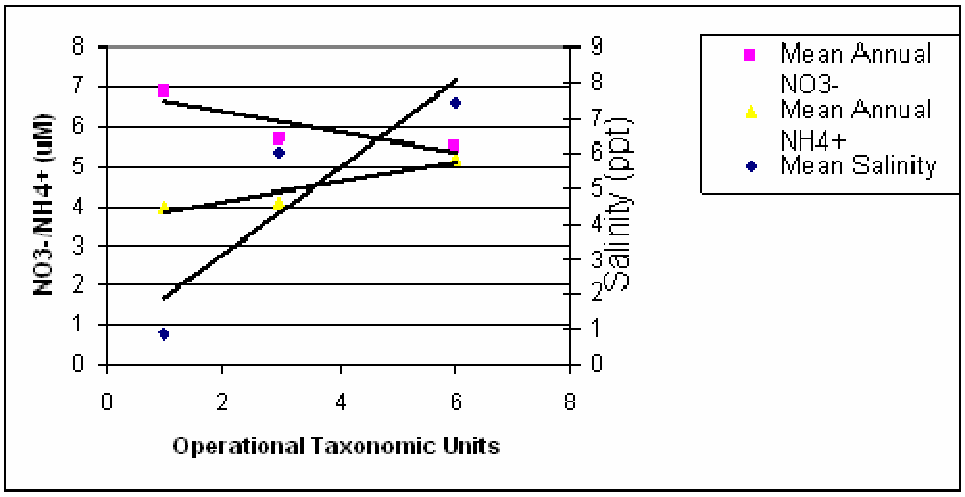

Figure 12. Comparison between annual mean for environmental parameters and anammox bacterial diversity at each sampling site

Again with the annual mean, ammonium and salinity were positively correlated with diversity. Nitrate levels and diversity were inversely correlated (Figure 12). No relationships between the annual mean of environmental parameters and anammox diversity were significant. Fluctuations in these environmental parameters were also taken into account. The standard deviation of the environmental parameters for November, December, and January were plotted against anammox OTU's from each sampling site (Table 6, Figure 13).

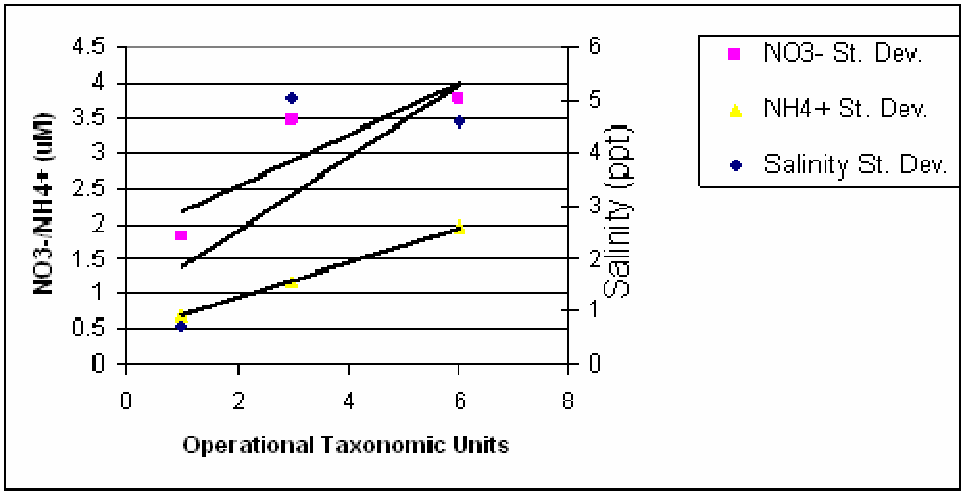

Figure 13. Comparison between standard deviation of environmental parameters for three months and anammox bacterial diversity at each sampling site

Fluctuation of all these parameters for the January samples was positively correlated with anammox diversity. The effect of the fluctuation of salinity and nitrate on anammox diversity was not significant. The effect of the fluctuation of ammonium on anammox diversity was extremely significant, with a p-value of 0.002. Annual fluctuations of these environmental parameters were also considered in this study (Figure 14). Salinity varied the most throughout the year at site M54 with a standard deviation of 5.1 ppt. The annual salinity at site M61 varied by 4.8 ppt, and site NAV had the lowest annual standard deviation for salinity, which was only 1.4 ppt . Ammonium also varied the most throughout the year at site M54, giving a standard deviation of 3.3 µM. The annual ammonium concentration at site NAV varied by 2 µM from the average for the year, and site M61 had the lowest annual standard deviation for ammonium, 1.6 µM. The annual standard deviation for nitrate was greatest at site NAV, 3.3 µM. Sites M61 and M54 had close to equal standard deviations for the annual nitrate concentrations, 2.5 and 2.6 µM, respectively (Table 1). By plotting the number of operational taxonomic units (OTU's) supplied by DOTUR analysis against the annual standard deviation of the environmental parameters of salinity, nitrate/nitrite, and ammonium levels for the three sampling sites, relationships between anammox bacterial diversity and variation of the environmental parameters for the year were determined (Figure 14).

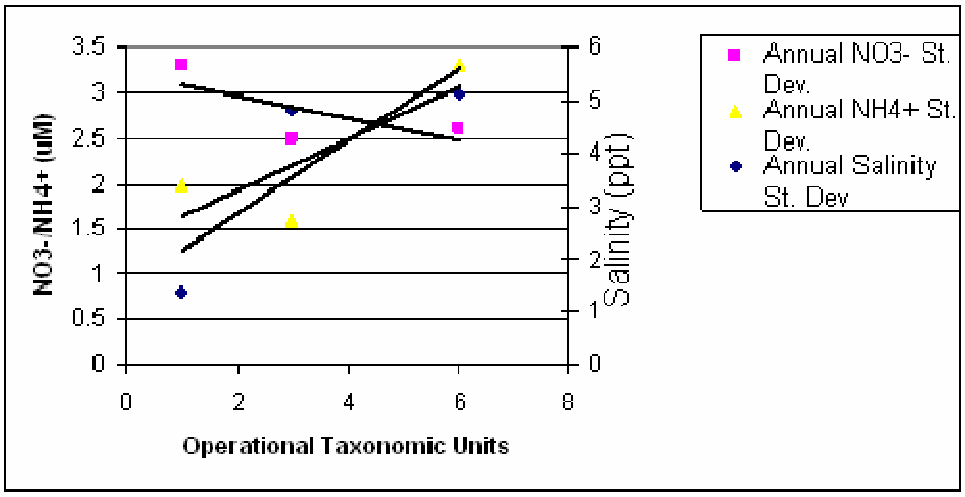

Figure 14. Comparison between annual standard deviation of environmental parameters and anammox bacterial diversity at each sampling site

There was a highly linear, positive correlation between change in salinity and anammox diversity and the change in ammonium concentration and annamox diversity. There was a negative correlation between change in the nitrate concentration and anammox diversity for these three sampling sites. However, the p-values for none of these relationships were significant.

Salinity seemed to have a substantial influence on diversity of anammox communities. The number of OTU's increases with salinity. *Brocadia* species dominated site NAV, with the lowest salinity (0.9 ppt) in the Cape Fear River Estuary. In sequencing data from the January sample and T-RFLP from 4 different months, *Brocadia* species was the only organism detected. The sites M61 and M54 with higher salinities are dominated by *Scalindua* species. A large *Kuenenia peak* was present in site M61 for the samples collected in the fall (Figure 9). *Scalindua* species have previously been identified in estuary systems, but this is the first discovery of *Brocadia*, *Anammoxoglobus*, and *Kuenenia* organisms in an estuary. *Brocadia* organisms were first discovered in a wastewater treatment plant, and were subsequently found, along with *Kuenenia* species, in large amounts in many bioreactors and waste treatment plants in (Schmid et al., 2005). *Brocadia* organisms have also been identified in the Ugandan wetland (Jetten et al., 2003). *Brocadia anammoxidans* and *Kuenenia stuttgartiensis* are considered freshwater anammox species (Jetten et al., 2003), and this agrees with the findings in this study. A wastewater treatment plant is nearer site NAV than any of the other sampling sites, and perhaps *Brocadia* and *Kuenenia* organisms detected in sites NAV and M61 originated at this wastewater treatment plant, but due to increased salinity have only been able to survive at these sites. Through a study with *Kuenenia stuttgartiensis,* it has shown that freshwater anammox bacteria can adapt to salt concentrations as high as 30 ppt, only if the salt concentration increases

gradually (Kartal et al., 2007b). Salinity values are the main environmental parameter that differ in these sites for the sampling dates used in this study (Table 2), and this may explain why detection of *Brocadia* organisms, especially the *Brocadia* community identified by the peak at 284 bp, decreased as sampling moved away from site NAV. *Kuenenia* organisms were detected in site M61 through sequencing of the January sample and T-RFLP analysis of the September sample. Therefore these organisms may have a niche requirement present at site M61 during the months of January and September, or they may be able to adapt to salinity changes more easily than *Brocadia* organisms.

CONCLUSION

This is the first report of the occurrence of anammox bacteria in the Cape Fear River Estuary. It provides a comparison study of the relationship between different anammox bacterial community composition and variations in environmental parameters by examining biogeographical distribution of anammox bacteria in the Cape Fear River Estuary along with seasonal and spatial monitoring.

Two anammox genera are rarely found in the same ecosystem implying that anammox organisms of different genera are niche specialists (Kartal et al., 2007b). Large phylogenetic distance between different genera of anammox bacteria gives further evidence that these organisms are specialized for different ecological habitats (Kartal et al., 2007a). Although, anammox organisms seem to be niche specialists, we discovered four distinct anammox communities, not only of different species but of different genera as well, in the same estuary. This biogeographical distribution exists over a very short distance in the Cape Fear River Estuary. The distance between site NAV and M61 is approximately three miles, and the distance between site M61 and M54 is approximately four miles. The main environmental parameter that differs between these sites is salinity. Annual ammonium and nitrate/nitrite levels between these sites are not that different (Table 1).

These results concluded that anammox bacteria thrive in environments of lower salinity, and that *Brocadia* and *Kuenenia* are freshwater adapted organisms. Four different anammox genera (*Brocadia*, *Scalindua, Anammoxoglobus*, and *Kuenenia*) were discovered in the same estuary of the Cape Fear River, and at site M61 three genera (*Brocadia*, *Scalindua,* and *Kuenenia*) were detected during the same sampling period. Microbial endemicity of anammox

bacterial communities was observed related to the changes in salinity. In the sampling site with the lowest salinity (NAV; 0.9 ppt) *Brocadia* organisms dominated at all sampling times, in the sampling site of intermediate salinity (M61; 6 ppt), *Kuenenia* and *Scalindua* communities seemed to coexist, and in the sampling site with the highest salinity (M54; 7.4 ppt), *Scalindua* organisms dominated at all times. The Cape Fear River Estuary offers a unique and important study site for anammox bacteria as proved by two differences discovered in this study as compared to anammox studies in other locations. First, *Brocadia* and *Kuenenia* organisms, that are mainly only detected in wastewater treatment systems, were detected in the Estuary. Second, three different genera were found to coexist during the fall season at site M61, the sampling site of intermediate salinity. The natural salinity gradient that exists in the Cape Fear River Estuary and the changes in this gradient affected by spatial and seasonal variation supplied an untreated study site to examine anammox community changes in their true environment.

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CHAPTER 3. ANAMMOX ACTIVITY IN CAPE FEAR RIVER ESTUARY SEDIMENTS

INTRODUCTION

Isotopes are forms of the same element differing only in the number of neutrons. All elements have multiple radio and/or stable isotopes. Because stable isotopes are not hazardous to human health, do not undergo radio-decay, or vary in reactive chemistry, researchers frequently use them to estimate various reaction rates (Fry, 2006). Stable isotope measurements permit detailed views of natural element cycling in systems of interest; these are usually systems involving the cycling of the following stable isotopes: carbon ($^{13}C/^{12}C$), nitrogen ($^{15}N/^{14}N$), sulfur ($^{34}S/^{32}S$), oxygen ($^{18}O/^{16}O$), and hydrogen ($^{2}H/^{1}H$) (Fry, 2006; Lajtha and Michener, 1994). Two different methods using stable isotopes in ecological research are natural abundance measurements and isotope tracer experiments. In natural abundance experiments, stable isotope ratios become useful to ecologists due to a predictable physical and enzymatic-based discrimination against heavier isotopes, a process known as fractionation. Samples containing more of the heavy isotope (ex., ^{15}N) are referred to as 'enriched' and are considered 'heavier' than samples containing more of the light isotope (ex., ^{14}N), which are referred to as 'depleted' or 'lighter'. Natural abundance experiments can provide clues to the origins of materials in the environment when potential sources are isotopically distinct from one another or they can provide indicators about mixing of source material through certain chemical or biotic processes when the fractionation rules are known for all reactions involved (Lajtha and Michener, 1994). Natural abundance isotope frequency cannot normally be used to estimate actual rate reactions.

Nitrogen isotope tracer techniques have been used for decades to quantify specific N-cycling reactions. Through incubations with the labeled isotope, ^{15}N, rate estimates for many important geochemical processes in the nitrogen cycle, such as nitrification (Tobias et al., 2001a; Tobias et al., 2003; Wessel and Tietema, 1992), denitrification (Nielsen, 1992; Steingruber et al.,

2001; Risgaard-Petersen et al., 2003), anaerobic ammonium oxidation (Risgaard-Petersen et al., 2003; Thamdrup & Dalsgaard, 2002), and dissimilatory nitrate reduction to ammonium (Tobias et al., 2001b), may be derived. Rates are calculated from the incorporation of ^{15}N label from the spiked substrate to the product (Glibert & Capone, 1993). The ^{15}N isotope labeling technique is especially useful for studying processes involved in nitrogen removal, denitrification and anammox, because each process combines nitrogen elements in a different way that can be tracked by amending samples of interest with differently labeled substrates. For reactions that produce N_2, such as denitrification and anammox, each reaction rate can be calculated according to how the ^{15}N is distributed into N_2 masses of 28, 29, and 30 amu's (Steingruber et al., 2001; Risgaard-Petersen et al., 2003). All possible combinations of ^{15}N distributions by each reaction are shown in Table 7.

Table 7. Possible N_2 productions for each treatment by N-removal reactions

(All enrichments are at the 99+ atm % level.)

Treatment	Reaction	
	Anammox	Denitrification
$^{15}NO_3^- + {}^{14}NH_4^+$	$^{29}N_2$	$^{30}N_2$
$^{15}NH_4^+ + {}^{14}NO_3^-$	$^{29}N_2$	$^{28}N_2$
$^{15}NH_4^+$	0	0*

*Unless O_2 contamination occurs, in which case $^{15}NH_4^+$ is oxidized to $^{15}NO_3^-$ through nitrification and $^{30}N_2$ is produced through coupled denitrification.

By carrying out these ^{15}N-tracer based rate measurements, an estimation of the rate of N removal and the reaction primarily responsible for this removal, denitrification or anammox, can be calculated for the microbial communities at the three sampling sites in the Cape Fear River Estuary. This method allows quantification of the contribution of anammox and denitrification to N_2 production. Because the environmental parameters were not manipulated in this experiment, these rates display the metabolic potentials of the anammox communities present at the site under optimal NO_3-, NH_4^+, and O_2 conditions. The rate of each reaction may be linked to the natural changes in salinity, nitrate, and ammonium levels that occur along the Estuary. In conjunction with earlier molecular work, a relationship between variation in specific N-cycling removal reactions and specific microbial communities may be linked. Overall, the objectives of this chapter are to measure activities of anammox and to determine the contribution of N_2 production by anammox and the relationship if any, of anammox activities to environmental parameters at the time of sampling or to the range of variation in these parameters for any given site in the Cape Fear River Estuary.

MATERIALS & METHODS

Sample Collection

Sediment was collected with a petite Ponar grab sampler from three sampling sites in the Cape Fear River Estuary (Wilmington, North Carolina), NAV, M61, and M54, in November 2006 and stored in the anaerobic chamber until the sediment incubations were run within three weeks of collection (Figure 15).

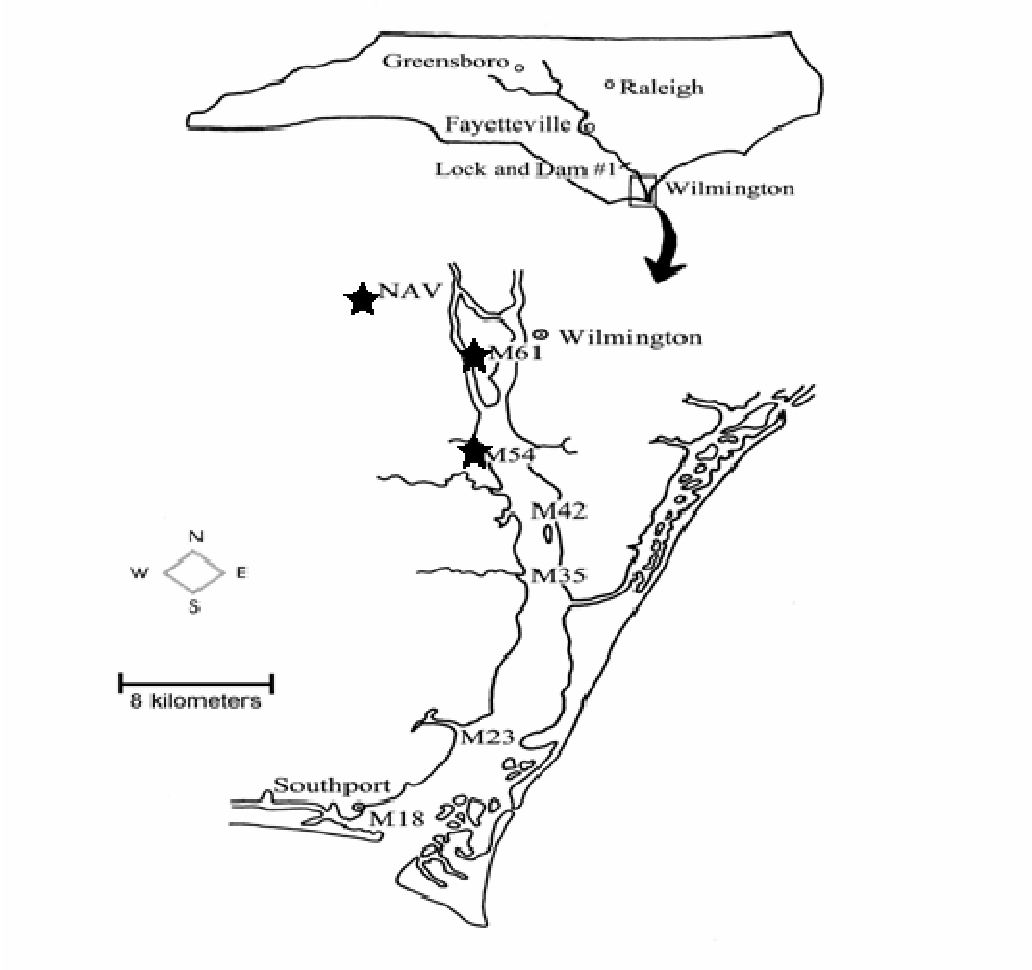


Figure 15. Map of sampling sites in the Cape Fear River Estuary

Sediment Incubations

To measure anammox and denitrification activity, approximately 2 g of wet sediment was removed from core samples and transferred to 12 glass vials (12ml), undergoing a constant flushing of helium, and sealed with gas-tight caps with septums (Exetainer, Labco). The 12 samples were left to stand overnight to eliminate the background concentration of NO_3- and NH_4^+ in the sediment. The following day, each vial was flushed with U.H.P. grade helium. Subsequently, 100µl of 99.0% helium-purged stock [N] solution of one amendment, i.e. (1) $^{15}NO_3$- plus $^{14}NH_4^+$, (2) $^{15}NH_4^+$ plus $^{14}NO_3$- or (3) $^{15}NH_4^+$, only was injected through the septa with a helium flushed syringe resulting in a final concentration of 100µM. An automated gas bench interface (Finnigan Gas Bench II) that was in line with a continuous flow isotope ratio mass spectrometer (IRMS) (Thermo Finnigan Delta V) was programmed to carry out a time course experiment, and sediment incubations were sampled at 0, 30, 45, 60, 75, 105, 120, 165, 180, 300, and 315 minutes. Blanks were distributed through the time course to correct data for any potential $^{29}N_2$ or $^{30}N_2$ added from vial leakage. The $^{15}NH_4^+$ treatment was done to ensure that there was no oxygen contamination contributing to $^{29}N_2$ production through coupled nitrification and denitrification, thereby artificially inflating the calculated anammox rate from mass $^{29}N_2$ accumulation. Anammox, denitrification, and the contribution of anammox to N_2 production were calculated from the production of ^{29}N and ^{30}N in the samples amended with $^{15}NO_3$- + $^{14}NH_4^+$.

Samples from sites M61 and NAV treated with $^{15}NO_3$- plus $^{14}NH_4^+$ were run in triplicate, and samples from site M54 treated with $^{15}NO_3$- plus $^{14}NH_4^+$ were run in duplicate. Rates from

each time point were averaged together. Anammox rates were calculated based on the 29 mass area, and denitrification rates were calculated based on the 30 mass area.

Samples treated with $^{15}NH_4^+$ plus $^{14}NO_3$- and $^{15}NH_4^+$ only were run once on the mass spectrometer. Leakage was corrected for in each sample by the 36 and 40 mass areas of argon (Ar) and known atm Ar/$^{28}N_2$, Ar/$^{29}N_2$, and Ar/$^{30}N_2$ ratios. Although, coupled nitrification and denitrification was very low, it was also corrected for by subtracting the production of ^{29}N and ^{30}N in the $^{15}NH_4$+ only treatments from the anammox rates in the $^{15}NO_3$- + $^{14}NH_4^+$ treatments, therefore these rates are conservative estimates.

$^{15/14}N$ Analyses and Calculations

The $^{29}N_2$ and $^{30}N_2$ were measured directly in the incubation vials. A simplified version of the equations by Thamdrup and Dalsgaard (2002) were used to quantify the pathways of N_2 production in the $^{15}NO_3$- + $^{14}NH_4^+$ treatment only. Equation (1) corresponds to N_2 production by anammox, and equation (2) corresponds to N_2 production by denitrification.

(1) $A_{28}=A_{total} * (1-F_N)$

F_N= 0.99 because this is the minimum value of our enrichment.

Therefore, $A_{28}=A_{total} * (1-0.99)$, which is 0.01 or 1%.

$A_{29}=A_{total} * F_N$

Therefore, $A_{29}=A_{total} * 0.01$

Defined Terms:

A_m represents the production of N_2 of mass m through anaerobic ammonium oxidation.

$A_{total}= A_{28} + A_{29}$

F_N= the fraction of ^{15}N in NO_3-, in this case 99%

(2) $D_{28}=D_{total} * (1-F_N)^2$

$D_{29}=D_{total} * 2 * (1- F_N) * F_N$

$D_{30}=D_{total} * F_N^2$

F_N= 0.99 because this is the minimum value of our enrichment

Therefore, $D_{28}=D_{total} * (1-0.99)^2$, which is 0.0001 or 0.01%

$D_{29}=D_{total} * 2 * (1- 0.99) * 0.99$, which is .019 or approximately 2%.

Defined Terms:

D_m denotes production of N_2 of mass m through denitrification.

$D_{total}= D_{28} + D_{29} + D_{30}$

This approach considered only $^{29}N_2$ production for anammox and $^{30}N_2$ production for denitrification in the $^{15}NO_3$- + $^{14}NH_4^+$ incubation. The simplification of these equations is possible because the $^{15}NO_3$- used is > 99% enriched, such that contributions of the 28 mass to anammox and the contributions of 28 and 29 mass to denitrification were negligible. This simplification was performed because measuring changes in 28 mass at such small levels against the large background of 28 was at the level of precision of the instrument and consequently uncertain. This simplification could potentially lead to an underestimate of anammox by 1% and underestimate of denitrification by 2%. Mass areas 29 and 30 were measured in the time course incubations as peak areas.

The rate calculations were performed as follows. Peak areas were converted to 29 and 30 masses according to known air calibration standards using the known atmospheric composition of 28, 29, and 30 N_2. The contribution of 29 and 30 mass N_2 from exetainer leakage in the incubations was corrected for by concurrently measuring changes in ^{40}Ar in blank exetainers

measured simultaneously by the IRMS. Argon areas in the blanks were converted to masses according to the calibrated air standards and converted to blank 29/30 masses for N_2 based upon accepted atmospheric ratios of $^{29}N_2$ to argon and $^{30}N_2$ to argon. A second correction was applied to the $^{29}N_2$ data to account for any possible effect of coupled nitrification and denitrification generating mass 29 in the $^{15}NO_3$- + $^{14}NH_4^+$ incubations. Collectively, leakage and coupled denitrification accounted for less than 3% of the observed 29 and 30 mass accumulations. Least square linear correlations were determined for the time course corrected 29 and 30 mass accumulations. The slope of the corrected 29 mass vs. time and corrected 30 mass vs. time correlations were accepted as the rates of anammox and denitrification, respectively (Figures 16, 17, & 18). To determine the percent significance of the anammox reaction to the total removal of N_2, the anammox rate was divided by the total N_2 removal rate, the anammox rate and the denitrification rate

RESULTS

Eight incubations of (5.25 hours each) were performed with the addition of $^{15}NO_3$- + $^{14}NH_4^+$, and production of $^{29}N_2$ (anammox) and $^{30}N_2$ (denitrification) was detected in sediments from all locations. Denitrification rates ($^{30}N_2$) were much higher than anammox rates ($^{29}N_2$) in each sampling site. Production of $^{29}N_2$ and $^{30}N_2$ was linear during the incubations with $^{15}NO_3$- + $^{14}NH_4^+$ (Figures 16, 17, & 18). The average of salinity, nitrate, and ammonium levels for September, October, and November from each sampling site (Table 8), in addition to salinity, nitrate, and ammonium levels for October are shown in Table 8. The average rates of all reactions for each sampling site and the contribution of the anammox reaction to total N_2 removal are shown in Table 9. The percent of N_2 production due to anammox ranged from 4.3 to 15.5%. Significant difference was found in anammox activity between sites M61 and NAV as determined by p-value (<0.05). There was however no significant difference in activity between sites NAV and M54 and sites M61 and M54 (Table 10).

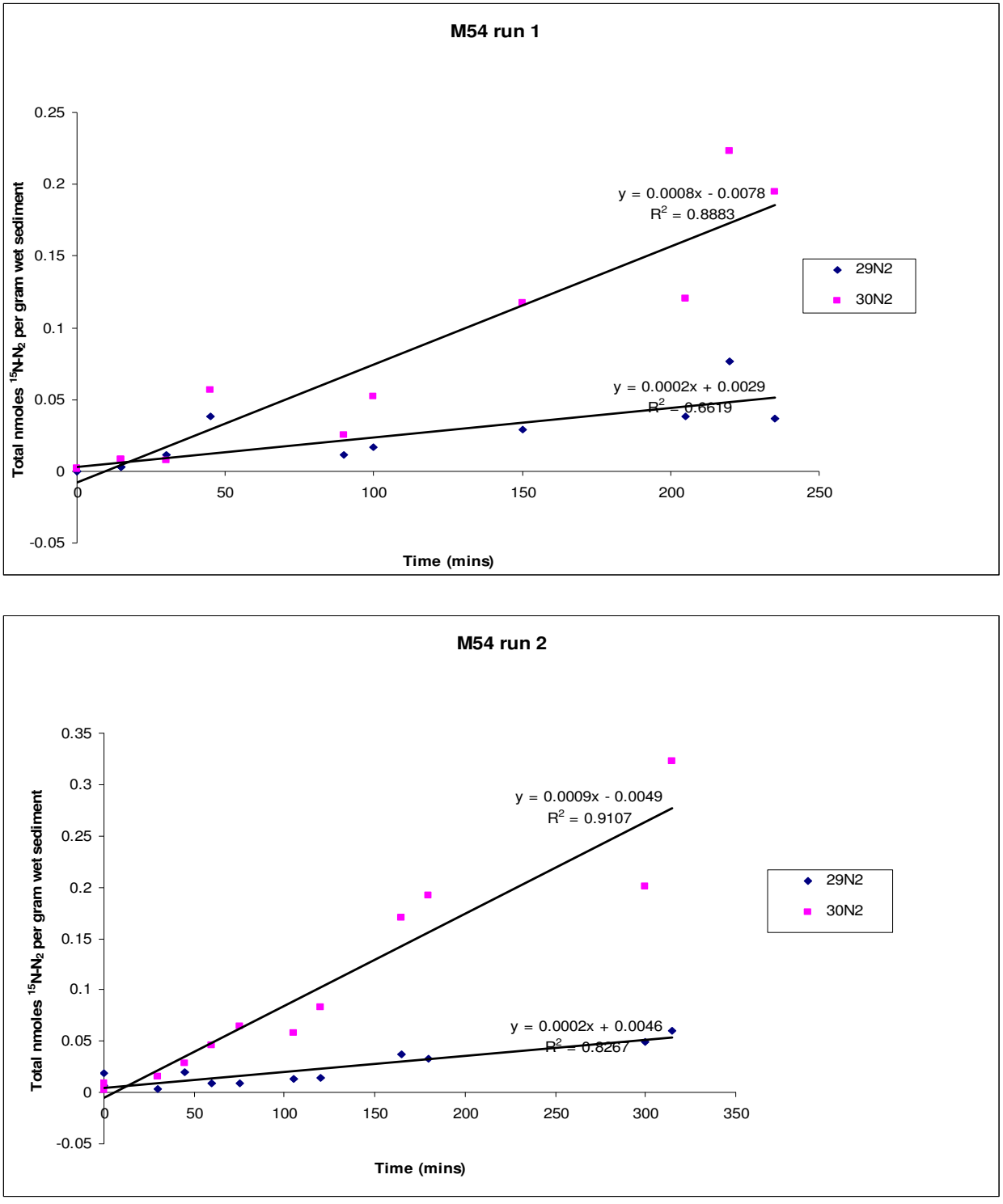


Figure 16. Production of $^{29}N_2$ and $^{30}N_2$ during incubations with $^{15}NO_3^-$ + $^{14}NH_4^+$ additions for site M54

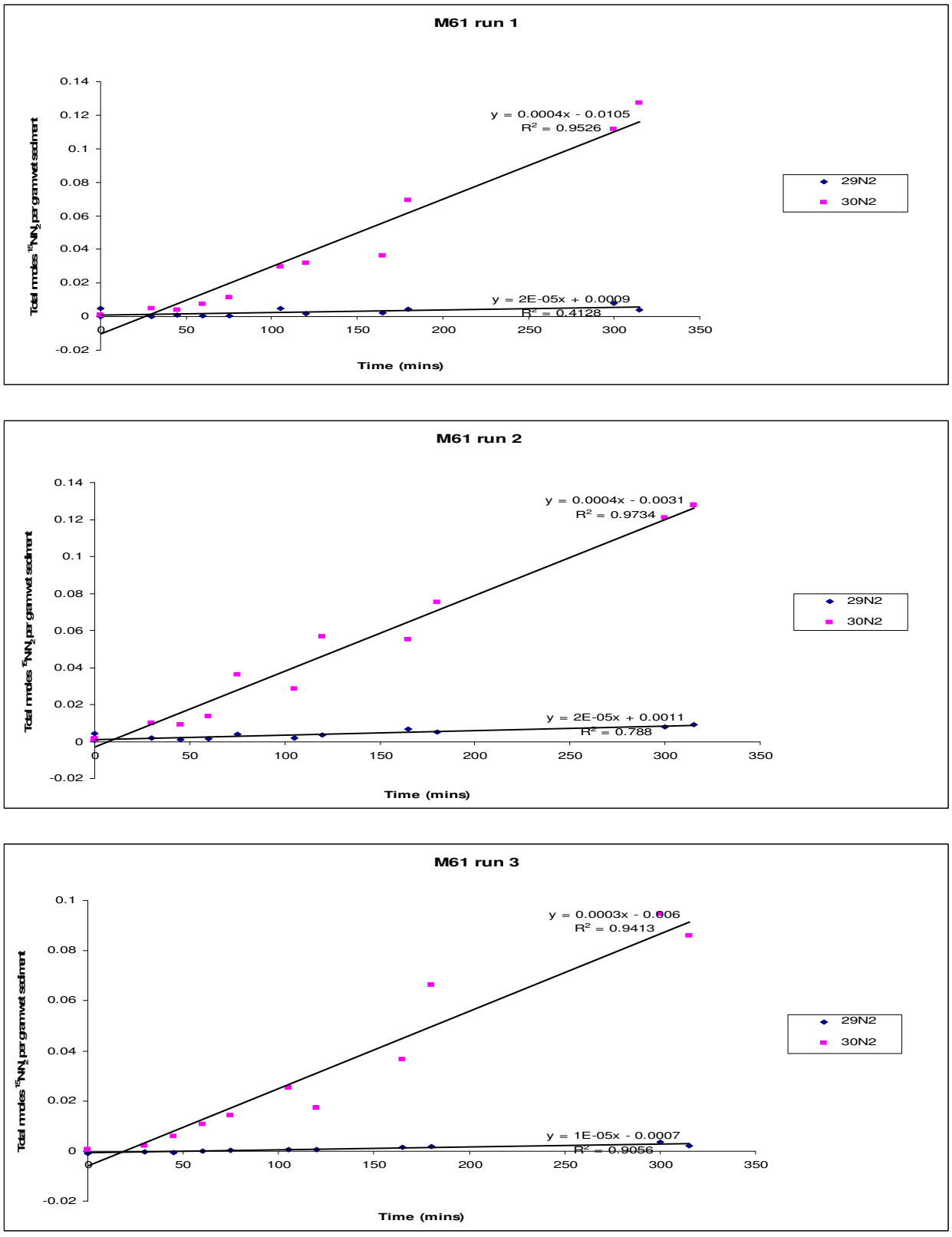
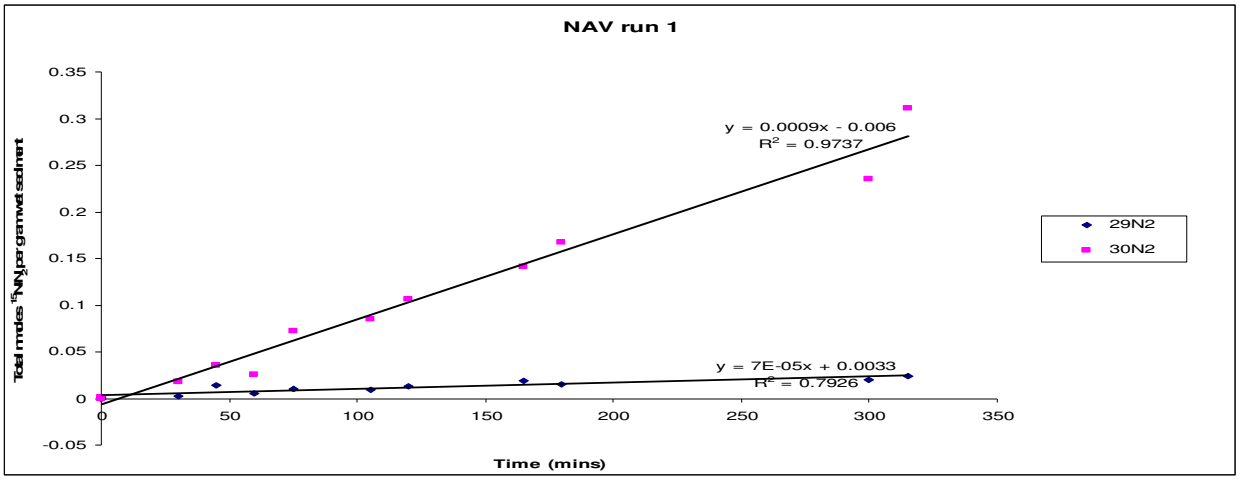
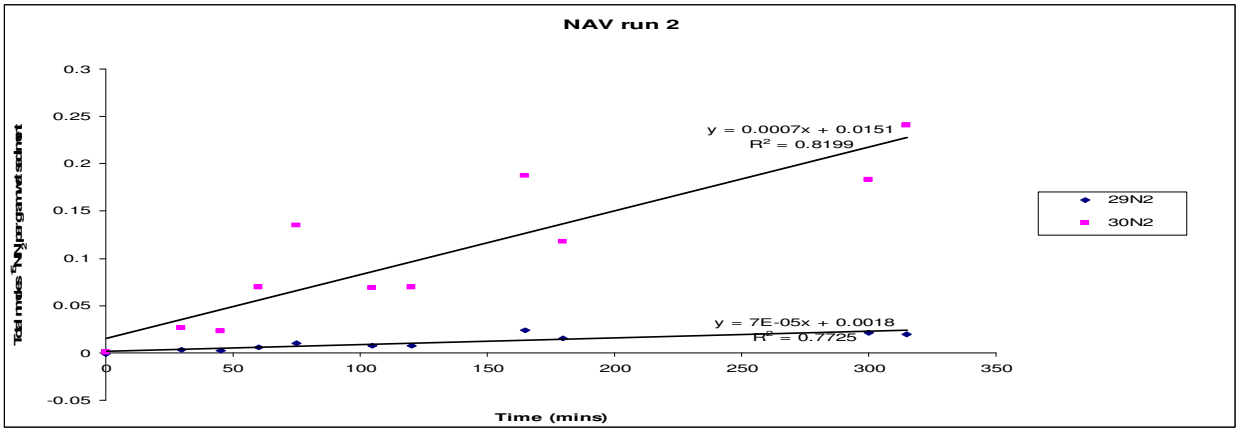


Figure 17. Production of $^{29}N_2$ and $^{30}N_2$ during incubations with $^{15}NO_3^-$ + $^{14}NH_4^+$ additions for site M61





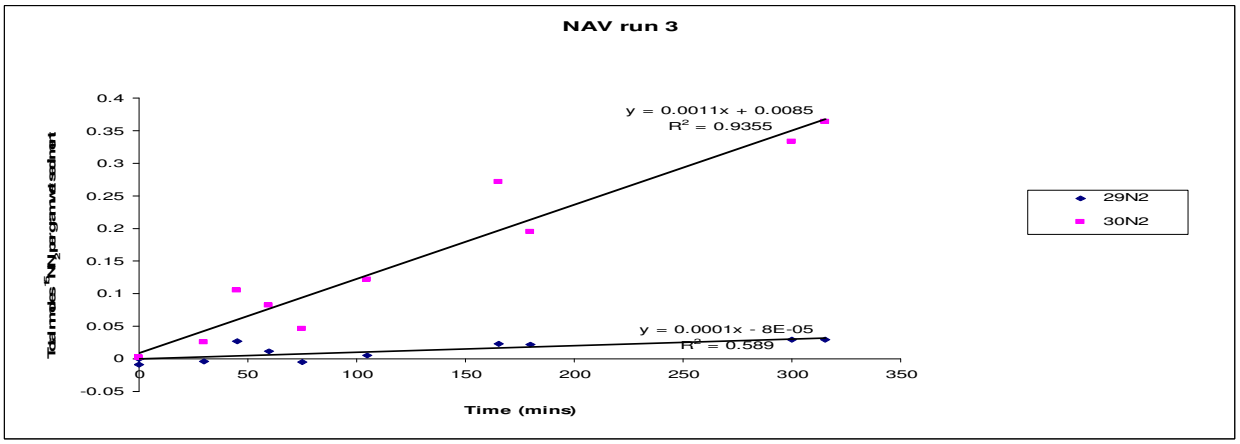


Figure 18. Production of $^{29}N_2$ and $^{30}N_2$ during incubations with $^{15}NO_3^-$ + $^{14}NH_4^+$ additions for site NAV

Table 8: Environmental parameters at each sampling site

Site	Mean Salinity (ppt)	October Salinity (ppt)	Mean Nitrate (µM)	October Nitrate (µM)	Mean Ammonium (µM)	October Ammonium (µM)
NAV	0.5 ± 0.78	0.1	4 ± 2.34	5.2	2.9 ± 1.15	3.3
M61	4.56 ± 5.38	10.5	2.67 ± 1.64	3.9	3.46 ± 1.13	4.4
M54	7.13 ± 5.86	11.1	2.4 ± 1.21	3.2	3.26 ± 0.56	3.8

Table 9. Endpoint activity measurements for each sampling site

Site	Anammox rate µmoles N per g wet sed hr^{-1}	p-value for anammox rate	R^2 value for anammox rate	Denit. rate µmoles N per g wet sed hr^{-1}	p-value for denit. rate	R^2 value for denit. rate	% of anammox/ total N_2 production
NAV	0.489 ± 0.118	0.002	0.718	5.22 ± 1.33	< 0.001	0.909	8.6
M61	0.097 ± 0.034	0.008	0.702	2.15 ± 0.323	< 0.001	0.945	4.3
M54	0.909 ± 0.212	< 0.001	0.744	4.94 ± 0.291	< 0.001	0.899	15.5

Table 10. Differences in anammox activity at each site represented as p-values

Site	NAV	M61	M54
NAV	–	0.015	0.119
M61	0.015	–	0.058
M54	0.119	0.058	–

NAV, the most freshwater site with lowest ammonium levels and highest nitrate levels, had anammox activity of 0.489 µmoles N per gram wet sediment hr^{-1}, with N_2 removal contributed to the anammox process at 8.6%. Site M61 of intermediate salinity, highest ammonium, and intermediate nitrate levels had anammox activity at 0.097 µmoles N per gram wet sediment hr^{-1}, with N_2 removal contributed to the anammox process at 4.3%. Site M54 of highest salinity, intermediate ammonium, and lowest nitrate had anammox activity at 0.909 µmoles N per gram wet sediment hr^{-1}, with N_2 removal contributed to the anammox process at 15.5% (Table 9). Denitrification rates were highest in site NAV intermediate in site M54, and lowest in site M61 (Figure 19, Table 9). Anammox rates were highest in site M54, intermediate in site NAV, and lowest in site M61 (Figure 20, Table 9).

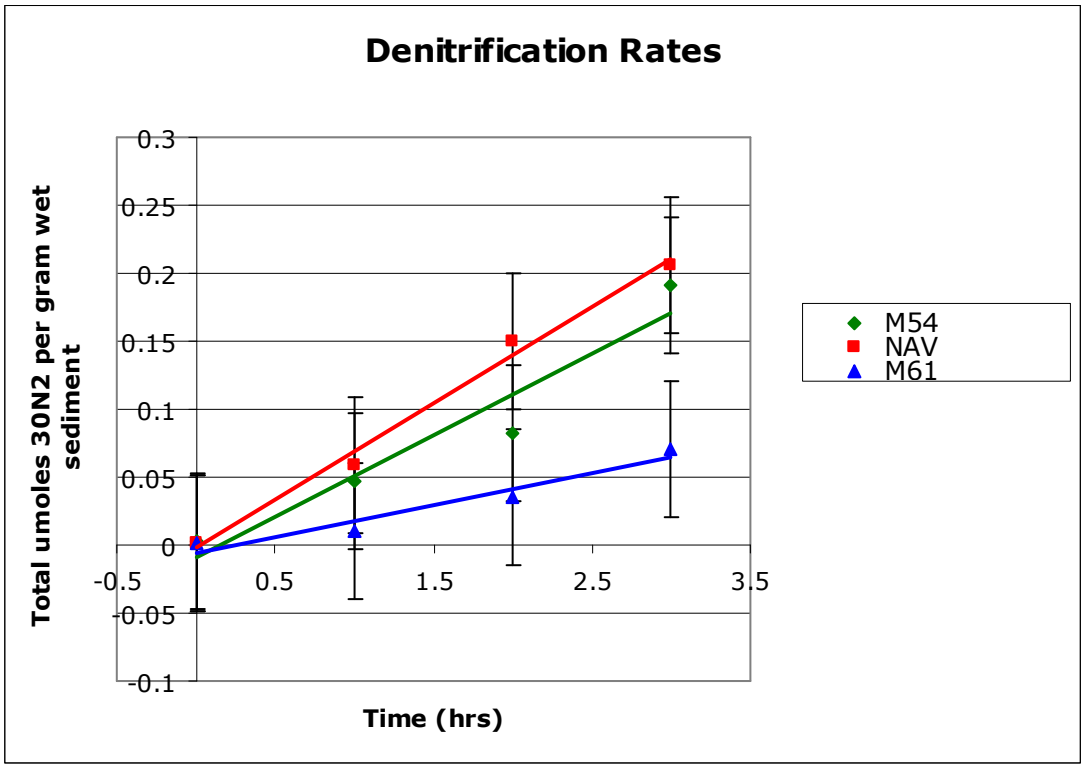


Figure 19. Denitrification rates in sediment incubations from each site

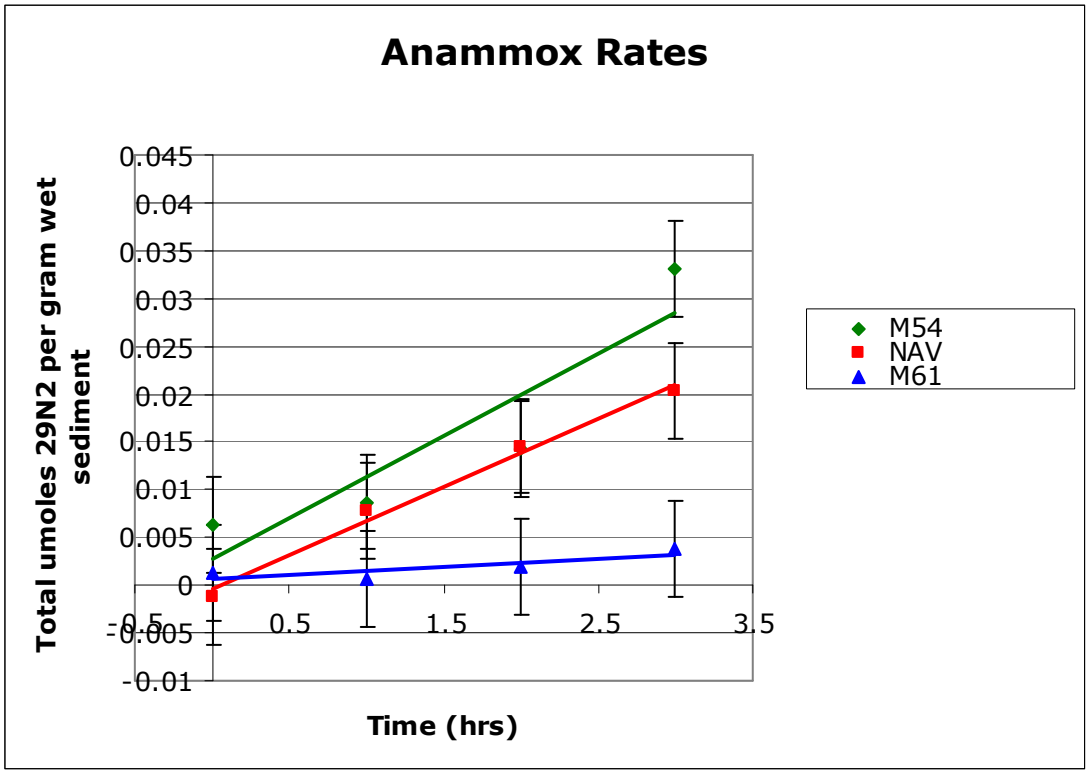


Figure 20. Anammox rates in sediment incubations from each site

The sediment used in the ^{15}N IPT experiments were collected in November, but because of the slow doubling time of anammox bacteria the environmental parameters of October were considered when testing for correlation between these environmental parameters and anammox and denitrification activity (Figure 21). No correlation could be established between salinity, nitrate, and ammonium levels and anammox or denitrification activity.

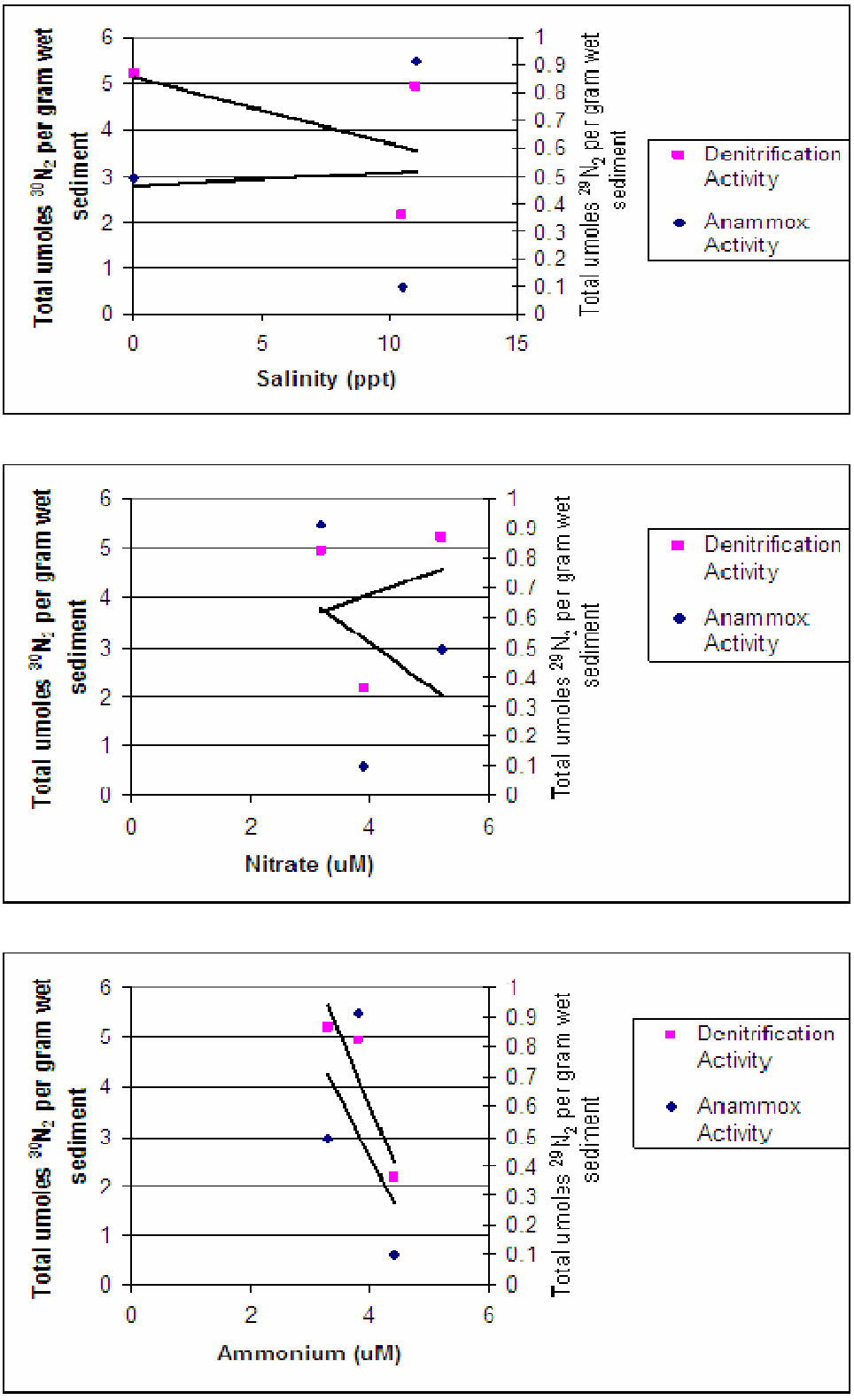


Figure 21. Comparison of environmental parameters and activity at each site

The standard deviation of these environmental parameters for the two months previous to sediment collection (September and October) and the month of collection (November) were also considered to examine whether these variations had any affect on anammox activity. No significant correlation was found between the fluctuation in environmental parameters and activity either (Figure 22).

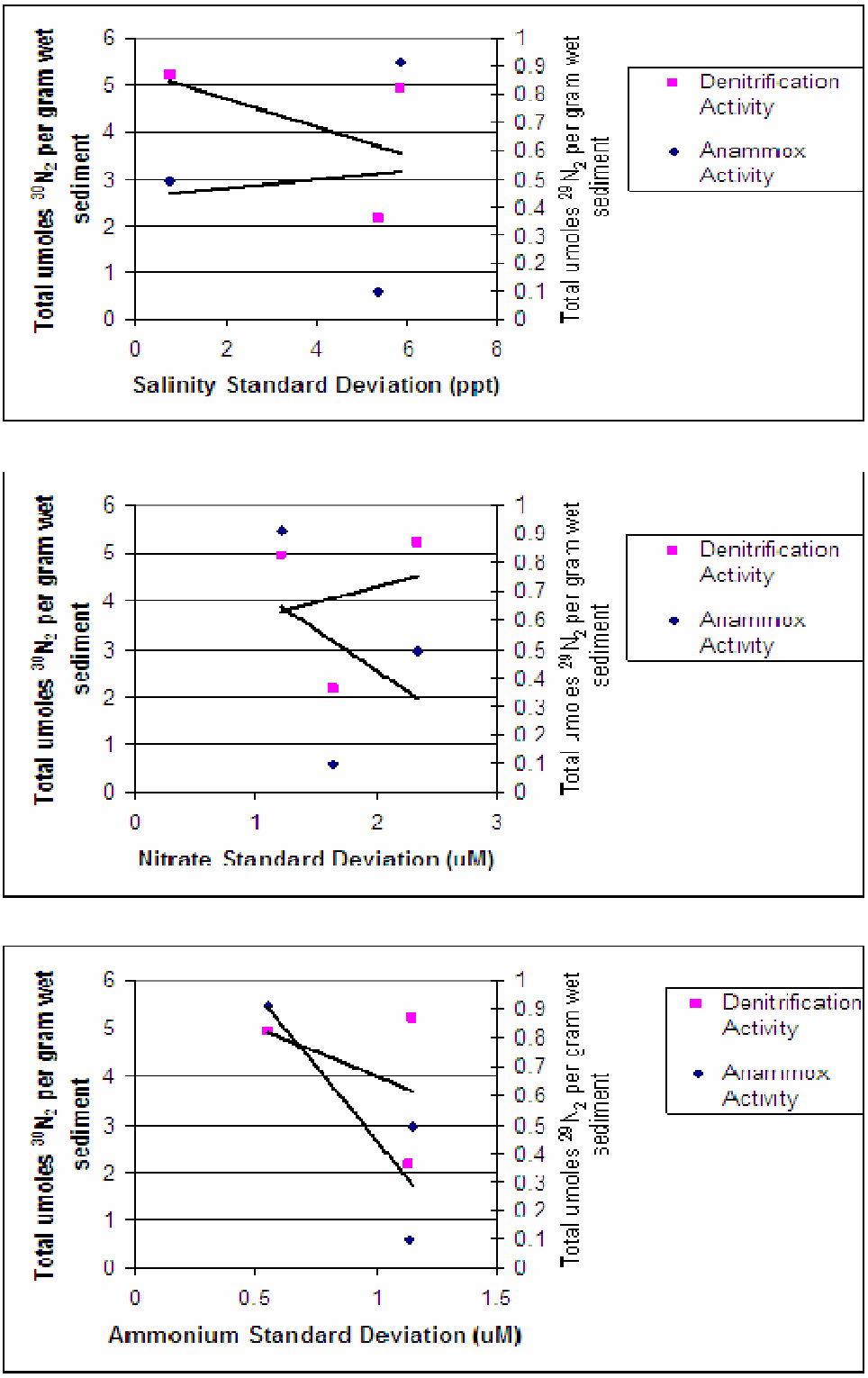


Figure 22. Comparison between standard deviation of environmental parameters for three months and denitrification and anammox activity at each sampling site

The levels of anammox based on $^{29}N_2$ production in $^{15}NH_4^+$ plus $^{14}NO_3$- additions were generally lower and not taken into account because of greater dilution of $^{15}NH_4^+$ by natural $^{14}NH_4^+$ present in the sample and its production during the incubations. The dilution was likely to be large, violated the $^{15}N > 99\%$ assumption for the calculations, and could not be measured due to small sample size.

DISCUSSION

Anammox activity was detected in sediment incubations from every sampling site. The total contribution of the anammox reaction to N_2 production ranged from 4.3% in site M61 to 15.5% in site M54. Anammox activity at site NAV was 8.6%. Denitrification exceeded anammox activity in every sampling site, which has been shown in other studies (Table 9). Furthermore, environmental parameters in different sampling sites were correlated to anammox and denitrifying acitivity (Table11).

Table 11. Summary of various environmental parameters on denitrification and anammox rates and community structure

Parameter	Denitrification Rate	Anammox Rate	Denitrification Community	Anammox Community	Reference	Measured in this Study
Organic Matter	+	O	+	-	Tal et al., 2006 Engstrom et al., 2005 Thamdrup & Dalsgaard, 2002 Risgaard-Petersen et al., 2004	N
Salinity	-	Unknown	-	Unknown	Sorenson, 1987; Yoshie et al., 2004	Y Water Only
NO_3- /NO_2	+	+	+	+	Cornwell et al.,1999; Herbert, 1999	Y Water Only
NH_4^+	+	+	O	+	Tal et al., 2006 Engstrom et al., 2005 Kuypers et al., 2005	Y Water Only

+= positive effect
O= neutral effect
-= negative effect

It has been suggested that higher organic carbon levels, remineralized solute production, benthic O_2 consumption, and surface sediment chlorophyll a levels promote denitrification over anammox activity (Tal et al., 2006; Engstrom et al., 2005). Higher organic carbon levels promote denitrification because it is an organotrophic process. Direct consequences of sediment oxygen consumption are an extended surface sediment layer where O_2 and NO_3- are detected in the pore water along with very high concentrations of solid phase manganese oxides. Elevated concentrations of reducing species seem to favor denitrification over anammox (Engstrom et al., 2005). Data from the literature about factors promoting and inhibiting anammox activity can be somewhat conflicting, and therefore; one objective of this study was to obtain a better understanding of factors affecting anammox activity by connecting anammox rate measurements with environmental parameters.

It is known that increased salinity decreases denitrifying community diversity and the rate of denitrification (Yoshie et al., 2004; Sorenson, 1987). Denitrification was highest in the study site of lowest salinity (NAV), but it was intermediate in the study site of highest salinity (M54), and lowest in the study site of intermediate salinity (M61). Salinity effects on anammox activity are unknown. However, in this study, the site of highest salinity (M54) had the highest anammox activity, the site of intermediate salinity (M61) had the lowest anammox activity, and the site of lowest salinity (NAV) had intermediate anammox activity. Other studies suggest that nitrate levels and fluctuations in nitrate levels affect anammox activity (Rysgaard et al., 2004).

A study in Arctic marine sediments found anammox to account for 19% of total N_2 production in stable, bottom layers of an ice floe. Anammox was undetectable in annual sea ice from the same area, suggesting that anammox bacteria are favored in sediments with steady

nitrate concentrations (Rysgaard et al., 2004). Anammox activity was intermediate in site NAV with the highest nitrate levels, lowest in site M61 with intermediate nitrate levels, and highest in site M54 with the lowest nitrate levels. Therefore no correlation between anammox activity and the environmental parameters of salinity, ammonium, and nitrate levels were found in sediment communities from the Cape Fear River Estuary (Figure 21). Site M54 had the lowest standard deviation from mean nitrate levels. Anammox activity was highest in site M54, but because anammox activity was the next highest in site NAV, the site with the highest nitrate fluctuations, no correlaton with nitrate fluctuations and anammox activity could be confirmed either (Figure 22).

Experiments carried out in the water column of Golfo Dulce, Costa Rica, demonstrated a tight coupling between ammonium, not nitrite, liberated during denitrification, and further transformation of this ammonium to nitrogen gas by the anammox process. It also states that anammox activity increases at greater depths because of an increase in ammonium levels (Kuypers et al., 2005). However, in this study, the site with the highest ammonium levels (M61) had the lowest anammox activity, and the site with the lowest ammonium levels (NAV) had intermediate anammox activity, therefore; no correlation with ammonium levels and anammox activity could be established. Physical factors, such as temperature, on the physiology and ecology of anammox and denitrifying bacteria must be considered as well. In a study performed in Arctic marine sediments it was discovered that the anammox reaction has a lower temperature optimum than denitrification, speculating that anammox may be favored in colder environments (Rysgaard et al., 2004). This may be another reason why the anammox reaction increases with an increase in water depth. Samples were taken on the same day from approximately the same

depth (1m) in this experiment at the same sampling time; therefore temperature was relatively equal at all sampling sites.

Anammox bacteria are very slow growing with a doubling time of at least eleven days, and it is theorized that stable nitrate conditions are required to maintain an active population. Whereas, denitrifying bacteria have a doubling time of hours and may adapt more readily to varying nitrate concentrations. Anammox activity was highest in site M54, where nitrate concentrations varied by only 1.21 µM during the sampling period, and denitrification activity was highest in site NAV where the annual standard deviation of nitrate was the highest among the sampling sites, 2.34 µM. Anammox bacteria favor an environment with a more stable nitrate supply, but denitrifying bacteria can survive in areas of greater nitrate fluctuation due to alternative electron acceptors. The fact that most denitrifying bacteria are organotrophic organisms that can use O_2 as an electron acceptor also makes them better suited for the fluctuating availability of O_2 and NO_{x^-} imposed by microalgae (Rysgaard et al., 2004). Anammox are slow-growing obligate anaerobes that can use only one electron acceptor, nitrite, for energy production. Therefore, anammox seem to be dominated by stricter conditions than denitrifying bacteria. This may explain why denitrification activity greatly exceeded anammox activity in every sampling site. Factors such as mineralization of carbon substrates and nitrate reductants may influence anammox rates as well.

Two previous studies focusing on anammox activity in marine sediments found the relative importance of anammox to be independent of pore water nitrate concentration, but mainly influenced by rates of carbon mineralization in surface sediments, nitrite availability, and temperature (Thamdrup & Dalsgaard, 2002; Engstrom et al., 2005). Easily accessible carbon supports denitrification, and anammox activity often increases with water depth because of the

decrease in easily degradable carbon and benthic carbon mineralization (Risgaard-Petersen et al., 2004). Organic matter abundance does not seem to substantially affect anammox rates (Thamdrup & Dalsgaard, 2002; Engstrom et al., 2005). However, a decrease in organic matter may decrease competition between heterotrophic denitrifying bacteria and autotrophic anammox bacteria, allowing greater anammox activity. Organic carbon levels were not measured in this study, therefore; no conclusion between its levels and denitrification and anammox activity can be concurred. Observations also suggest a general competition between reductants for nitrite in the pore water, and other oxidants such as manganese oxides are important for absolute and relative rates of anammox in coastal marine sediments as well (Engstrom et al., 2005). No measurement of manganese oxides or other oxidants were performed, and only nitrate levels, not nitrite levels, were measured in this study.

Trimmer et al. found anammox to be dependent on the initial reduction of nitrate to nitrite by the total nitrate reducing community, suggesting nitrite availability to not be a problem because anammox communities have a high affinity for nitrite (<10μM), thus taking proportionately more nitrite at lower concentrations (Trimmer et al., 2005). Their data further suggests that some anammox bacteria may actually be coupled physically and metabolically to members of denitrifying communities and are therefore governed by the metabolism of heterotrophic nitrate and nitrite reducers. In this study, the site with the highest nitrate levels (NAV) had the highest denitrification activity, and the site with the lowest nitrate levels (M54) had the highest anammox activity. If there were complete coupling between the metabolism of denitrifying and anammox bacteria, it would be expected that denitrification and anammox activity would be highest at the same site and lowest at the same site. This suggests that the two reactions are not dependent upon one another but perhaps in competition. Site NAV may have

the highest dissolved organic carbon level promoting higher denitrification activity, and site M54 may have a combination of factors such as lower dissolved organic carbon and optimal nitrate, ammonium, or salinity conditions for the anammox reaction. If this is the case the group that is metabolizing at a higher rate may be determined by a suite of environmental parameters encompassed in the sampling site. Site M61 had both the lowest denitrification and anammox rates, however, the highest rates for each of the reactions occurred at different sites. Because anammox and denitrification activity were both the lowest in site M61, perhaps a factor such as dissolved O_2, which negatively affects both reactions, is highest at this site.

This data suggests no coupling between the metabolism of denitrifying and anammox bacteria and no relationship between the mean and fluctuations of environmental parameters (Figures 21 & 22). Anammox activity was highest in the site of highest salinity, but it was not lowest in the site of lowest salinity. Anammox activity was highest in the site of intermediate ammonium, but it was not lowest in the site of lowest ammonium. This suggests that salinity and ammonium in combination with many other factors may affect anammox activity. Many environmental parameters co-vary in the Cape Fear River Estuary proving it hard to determine a specific set of parameters that promote or deter anammox activity. Although, no correlation was found between the measured environmental parameters, and anammox activity, there was some interesting links between anammox activity and data derived from molecular analysis.

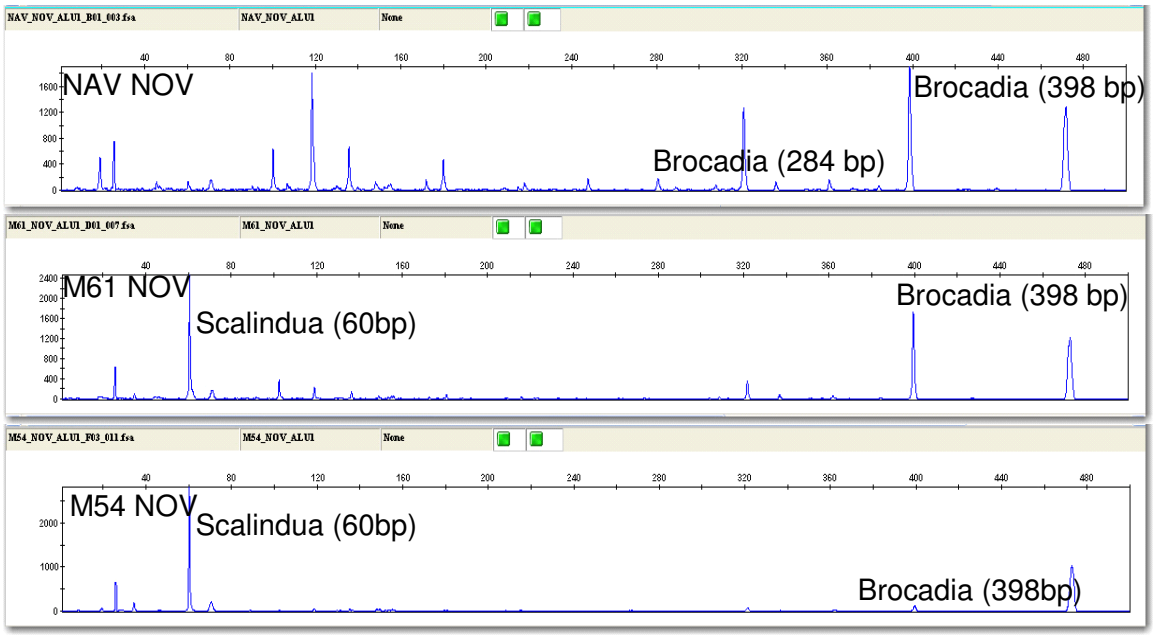


Figure 23. T-RFLP of anammox bacterial 16S rRNA genes for each site in November samples

The community profile of each site for the November samples, from which the activity measurements were performed, is shown in Figure 23. Site M54 with the highest anammox activity was dominated by *Scalindua* species, represented by the peak at 60 bp, a very small *Brocadia* peak was present as well at 398 bp. *Scalindua* bacteria may be more adapted to natural environments than *Kuenenia* and *Brocadia* organisms. This may explain why they are mainly detected in natural environments rather than wastewater treatment plants (Kuypers et al., 2003; Risgaard-Petersen et al., 2004, Kirkpatrick et al., 2006, Schubert et al., 2006, Rich et al., 2007). *Scalindua* bacteria have been detected in areas of very high anammox activity, such as the Black Sea (Kuypers et al., 2003). *Brocadia* and *Kuenenia* are mostly associated with wastewater treatment plants (Schmid et al., 2005). A physiological advantage in a natural system may promote a stronger metabolism and allow greater activity than that of less adapted anammox organisms. Site M61 was also dominated by *Scalindua* species, but a large *Brocadia* peak (398 bp) was also detected. This site had the largest mixed community present. Although *Brocadia* was detected at site M54, the peak was very small in comparison to the *Scalindua* community. In site M61, the *Brocadia* peak was almost as high as the *Scalindua* peak. Competition between these two communities could lower the overall N_2 productivity at this site. Site NAV was dominated by *Brocadia* organisms, which were first discovered in a wastewater treatment plant, and were subsequently found, along with *Kuenenia* species, in large amounts in many bioreactors and waste treatment plants in (Schmid et al., 2005). This is the first time that *Brocadia* organisms have been detected in an estuary, they have been detected in natural systems before.

Therefore, N_2 productivity was higher in the two sites that were dominated by just one anammox community. It was highest in site M54, where *Scalindua* organisms were detected in the highest number, intermediate in site NAV where only *Brocadia* species were detected, and lowest in site M61 where there was a mixed community of *Scalindua* and *Brocadia* organisms. Three wastewater treatment plants enter discharge into the Cape Fear River Estuary system. The Northside wastewater treatment plant empties into Smith Creek which enters into the northeast Cape Fear River above Wilmington, this is closest to site NAV. Downstream of NAV, closer to site M61 is the Town of Belville wastewater treatment plant. These two wastewater treatment plants could explain the source of *Brocadia* organisms present at sites NAV and M61. However, the Wilmington Southside wastewater treatment plant is adjacent to site M54. Very few *Brocadia* organisms were detected here. Because these are freshwater adapted organisms the high salinity at site M54 may not allow them to persist at this site.

CONCLUSION

Denitrification and anammox are the two processes in the nitrogen cycle that remove biologically available nitrogen and convert it into inert nitrogen gas, N_2. The availability of nitrogenous nutrients controls biological productivity in aquatic systems, and because primarily microbes catalyze the removal of nitrogen, it is important to understand the factors that influence the activity and diversity of denitrifying and anammox bacteria. The environmental parameters of salinity, ammonium, and nitrate levels were measured to derive correlations between these parameters and anammox community structure and anammox activity rates. For the activity study, samples were taken from three sites (NAV, M61, and M54) in the Cape Fear River Estuary that vary in these environmental parameters. Therefore, the community and its metabolic state was previously determined by a suite of physical, chemical, and biological controls encompassed in these environmental parameters. These factors were not manipulated in the experiment, and therefore reflected the communities' natural setting. To test the metabolic readiness of these communities to produce N_2 gas, each sediment incubation was prepared in such a way that nitrogen and oxygen conditions were optimal. In this study, anammox activity was highest in site M54. Site M54 has the highest salinity, the highest ammonium levels, and the lowest nitrate levels of all the study sites. At all sampling times, *Scalindua* organisms dominated. Site NAV has the lowest salinity, the lowest ammonium levels, and the highest nitrate levels of all the study sites. At all sampling times it was dominated by *Brocadia* organisms. At site NAV denitrification activity was greatest, and anammox activity was at an intermediate level. Site M61 was added to this study because its salinity, nitrate, and ammonium levels lie between those of sites NAV and M54. However, it has the lowest anammox and denitrification activity. The community profile of this site differed from that of the other two sites, NAV and M54, because

two different genera were distinguished by peaks generated through T-RFLP analysis. The other two sites were dominated by one genus only. Competition between the two different genera may explain the overall low anammox activity measured at this site. No correlations were found between environmental parameters and anammox activity. Therefore, this study provided more evidence for a connection between community structure and anammox activity than environmental parameters and anammox activity. This may be attributed to the fact that environmental parameters co-vary along the length of the Estuary or a bottom-up effect: environmental parameters determine the type of anammox communities present and the communities present determine the communities' activity levels at each site.

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BIOGRAPHICAL SKETCH

Olivia R. Dale was born on July 11, 1981, in Leland, Mississippi. She took summer classes in Belize in 2002, and this trip persuaded her to pursue a degree in marine biology. She graduated from the University of Mississippi in May 2003, with a B.A. degree in Biology. Upon graduation, she worked for one year as a research technician in a neurosurgery lab at the University of Alabama at Birmingham. Excited by the idea of combining research and further learning of marine biology topics, she entered the Marine Biology graduate program at the University of North Carolina Wilmington in August 2004. She worked under the direction of Dr. Bongkeun Song in the microbial ecology lab at the Center for Marine Science. Upon graduation in May 2007, Olivia will move to Dubuque, Iowa, and work as a medical microbiologist for United Clinical Laboratories, Inc.